CULTIVATING *PYCNANTHEMUM* FOR LANDSCAPE USE AND POLLINATOR SUPPORT

by

KAITLIN SWIANTEK

(Under the Direction of John Ruter)

ABSTRACT

Breeding for pollinators and consumers fosters public interest in pollinator conservation. *Pycnanthemum* is a pollinator-plant with market potential. The first study discussed in this work compared pollinator attractiveness between three *Pycnanthemum* species. *Pycnanthemum virginianum* attracted the most pollinators overall, while most pollinator categories preferred *P. flexuosum*. A second study explored the effect of paclobutrazol treatments. *Pycnanthemum virginianum* treated with 4 – 8 mg a.i./pot were shorter than the control. *Pycnanthemum flexuosum* treated with 6 mg a.i./pot were more compact, though flowering decreased. The final study determined the genome size of 14 *Pycnanthemum* species ranged from 2.89 pg (*P. californicum*) to 7.22 pg (*P. torreyi*). The study also found that ploidy and chromosome number could predict the genome size of *Pycnanthemum* species. Additional studies comparing propagation, phenotypes, mutagen dosages, and crosses provide beneficial information. Introducing a pollinator-plant that consumers covet encourages the supplementation of pollinator habitat and resources in urban landscapes.

INDEX WORDS: *Pycnanthemum*, genome size, flow cytometry, plant breeding, paclobutrazol, pollinators, native perennial

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DEDICATION

To my family

To my brother, Justin

I know you'd be proud of me

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The Significance of Pollinators and Stopping Their Decline

Pollinators are crucial to the ecosystem, human health, and the economy. Pollinators are integral to the environment because they act as an ecological bridge and influence multiple trophic levels (Bailey, 2022; Ollerton, 2021). The role pollinators provide as ecological bridges indicates that pollinators serve as prey to predators while pollinating the plants that herbivores consume (Ollerton, 2021). Their role in many food chains supports other plant and animal species, conserving biodiversity and ecosystem stability (Bailey, 2022; Porter, 2010). A variety of herbivores rely on pollination services for their diet; therefore, predators also rely on pollinators to support prey populations. Encouraging gene flow and reproduction are functions of pollinators, who are responsible for pollinating 87.5% of flowering plants (Bendel et al., 2019; Ollerton, 2021). Without pollination promoting reproduction and genetic diversity, many flowering plant species would become extinct or inbred. Inbreeding depression may be the result of excessive inbreeding and increases the probability of inheriting harmful or lethal alleles (Frankham et al., 2002). The extinction of plants and reduction of plant fitness limits the vegetation available to wildlife. Similarly, pollinators assist in crop production by encouraging reproduction in the plants humans consume and feed to livestock. Globally, pollinators are responsible for $235 – $577 billion worth of crop production each year (IPBES, 2016). Certain pollinators also provide services that reduce pesticide use, which saves money and decreases chemical pollution. For example, some Hymenoptera and Diptera pollinators may provide pest

control through predation or parasitism (Amarasekare, 2020; Proctor & Yeo, 1973). Approximately $4.5 billion worth of pest control is accomplished by pollinators annually in the U.S. (Losey & Vaughan, 2006). Without the pest control services provided by pollinators, pest pressure would be more intense, leading to increased pesticide use and production costs.

Despite the crucial role of pollinators in our society, pollinator populations have been declining globally. An increase in agriculture, change in land use, and loss of resources have reduced pollinator populations (Potts et al., 2010). Habitat fragmentation caused by urbanization can negatively impact pollinator habitats by removing resources and disrupting resource webs (Rhodes, 2018). Disrupting these webs can isolate pollinators, distancing them from resources and into unfamiliar territories (Steffan-Dewenter et al., 2006). Pollinators rely on a well-connected resource web and have a limited range in which they can detect the scent of floral rewards (Kearns et al., 1998). If fragmentation separates pollinators too far from nectar or pollen sources, they become isolated. Isolation makes populations susceptible to inbreeding and sudden extinction events (Steffan-Dewenter et al., 2006). Increasing floral spaces within urban areas could support pollinators by providing additional and more diverse resources.

Connecting pollinator habitats by introducing flowering plants in ditches, hedgerows, and medians could combat habitat fragmentation. Linear plantings in landscapes help pollinators locate and navigate between resources (Ollerton, 2021). Cranmer et al. (2012) observed how hedgerow connections between *Salvia pratensis* patches affected bumblebee and hoverfly visitation. The study examined *S. pratensis* patches arranged in equilateral triangles with two patches connected by natural or artificial hedgerows (Cranmer et al., 2012). Designing triangular patches with only one side connected by a linear landscape component allowed researchers to compare the visitation to connected plants against unconnected patches, which act as the control.

Cranmer et al. (2012) found significantly more pollinators visited plants connected by natural and artificial hedgerows, with a positive correlation between total visitors and connectedness ($r = 0.68$). The significant impact of the artificial hedgerow on visitation also showed that pollinators use linear landscape features to navigate between flower patches and are not simply foraging along hedgerows (Cranmer et al., 2012). These results allow a deeper understanding of the response of bumblebees and hoverflies to hedgerows and other linear landscape components. The pollinators use linear features to actively locate plants instead of merely stumbling upon them while feeding. Therefore, connecting pollinator habitats within a small radius may not require flowering plants, although pollinators could benefit from additional resources linking more distant patches.

Efficiently designing landscapes to guide pollinators to resources will ensure pollinators are supported within urban areas. For example, increasing linear resources and flower patches within an urban landscape would help pollinators navigate the city and travel between habitat fragments (Menz et al., 2011). Additionally, introducing generalist plant species into urban areas would provide a more stable habitat for pollinators and encourage stronger network interactions over time (Zografou et al., 2020). Generalist plants are accessible to a wider variety of pollinators than specialists, encouraging more pollinators to forage. Battle et al. (2021) compared the pollinator visitation of plant patches with different resource levels and plant diversity to determine which parameters attracted pollinators. The study observed a greater abundance and diversity of pollinators within plots with higher resource availability, demonstrating that pollinators prefer abundant resources rather than plant diversity. Many pollinators require nectar and pollen throughout their lifecycle. Bees collect pollen and nectar to feed larvae, collecting an amount dependent on the body size and species of the insect (Müller et al., 2006). Providing

pollinators with resource-rich plants will limit the foraging distance pollinators must fly and improve fitness (Klein et al., 2017). In a harsh urban environment, restoring pollinator habitat on roadsides or introducing small plantings helps mitigate the negative effects of habitat fragmentation.

Pollinator plants also provide the public an opportunity to engage in pollinator conservation. Cultivating pollinator plants to appeal to a broader target audience would educate consumers and garner support for pollinator conservation. Khachatryan et al. (2017) tracked the eye movement of 104 plant buyers to determine where participants' attention was focused when buying plants. Plants had labels related to pollinator plant status, production method, origin, plant type, and price (Khachatryan et al., 2017). Khachatryan et al. (2017) found that 47.7% of participants focused on pollinator plant signage and were more likely to purchase the plant due to signage. The study shows that consumers are interested in supporting pollinators, and there is a market for pollinator-friendly plants. A separate study surveyed 841 people with home landscapes, asking about their landscape plants and management (Campbell et al., 2017). The study found that 50% of participants with pollinator plants in their landscape also reduced pesticide use in their yards, while only 21% of participants who did not have pollinator plants limited their pesticide use (Campbell et al., 2017). The study suggests people become more aware of pollinators within their landscape when using pollinator plants, which participants reflected in their landscape management.

Companies have been using pollinator plants to address the climbing interest in purposeful plants and pollinator conservation. However, many companies certify current commercial varieties as pollinator plants instead of introducing new plants to the market. One reason for the focus on existing plants could be that people are unaware of the diversity of

pollinator plants. One survey, directed toward 114 horticulture employees, asked participants to name at least four pollinator plants (Westerhold et al., 2018). The plants mentioned most were *Asclepias*, *Echinacea*, *Aster*, and *Buddleia*, with 14%, 8%, 5%, and 5% of participants naming the plants, respectively (Westerhold et al., 2018). The horticulture employees involved in this study did not have a diverse list of pollinator plants, indicating consumers are also not exposed to the true diversity of pollinator-friendly plants. As demonstrated by the limited knowledge of the employees, companies focus on a few popular cultivars, which results in fewer introductions of new genera to the public.

Cultivating *Pycnanthemum* for Pollinators and the Public

Pycnanthemum is a pollinator-attractive plant with market potential, offering pollinator resources and landscape-worthy traits. The Xerces Society states that *Pycnanthemum* is attractive to an abundance and diversity of pollinators (Wheeler, 2017). The University of Georgia State Botanical Garden also considers *Pycnanthemum* a pollinator plant, naming the genus one of the Pollinator Plants of the Year in 2022 (The University of Georgia State Botanical Garden, 2022). Introducing *Pycnanthemum* to the pollinator plant market would benefit pollinators by providing plant diversity and additional resources. Previous research has quantitatively compared the number and diversity of pollinators visiting *Pycnanthemum* to other genera. One study observed and netted pollinators on plants of various genera to determine flower preferences of crop-pollinating and rare bees (MacLeod et al., 2020). MacLeod et al. (2020) found that *P. tenuifolium* attracted quadruple the crop-pollinating bees compared to *Asclepias tuberosa* and twice the number of rare bees as *Solidago rigida* (MacLeod et al., 2020). *Asclepias tuberosa* and *Solidago rigida* were the second most visited plants by crop-pollinating and rare bees, respectively (MacLeod et al., 2020), indicating *P. tenuifolium* attracted significantly more bees overall.

Cadotte et al. (2017) compared the pollinator attractiveness of 17 plants, including *P. tenuifolium* and *P. virginianum,* to explore whether plant diversity and phylogenic distance between plants could accurately predict the ecosystem functions of the plants. The study found pollinators preferred the *Pycnanthemum* clade, although no significant relationship existed between the phylogeny or diversity of plants and the attractiveness to pollinators (Cadotte et al., 2017). The *Pycnanthemum* species in the study were discussed as a single clade rather than compared independently for pollinator attractiveness; therefore, the differences in pollinator abundance and diversity between the species remain unreported.

Pycnanthemum (commonly referred to as mountain mint) is an herbaceous perennial member of the *Lamiaceae*. Native to North America, 18 of the *Pycnanthemum* species are located in the central and eastern U.S., including *P. albescens*, *P. beadlei, P. clinopodioides, P. curvipes, P. flexuosum, P. floridanum, P. incanum, P. loomisii, P. muticum, P. nudum, P. pilosum, P. pycnanthemoides, P. setosum*, *P. tenuifolium*, *P. torreyi*, *P. verticillatum*, and *P. virginianum* — while the last species, *P. californicum,* is located in California (Chambers, 1993). The genus is characterized by densely clustered inflorescences with small, tubular, white, or purple flowers. The simple, opposite leaves range from ovate to lanceolate. When bruised, the leaves emit a fragrance similar to mint or thyme. *Pycnanthemum* is protandrous, meaning the stamens mature before the stigmas are fully functional (Chambers & Chambers, 1971), which limits self-pollination. However, the clustered flowers are capable of pollinating each other. Thus, *Pycnanthemum* is a self-compatible plant, but the different maturity rates of sexual structures encourage hybridization. Individual species can be identified morphologically using Weakley's *Pycnanthemum* key (2020) and Chambers' dissertation on *Pycnanthemum* (1961b). However, morphological identification can be difficult due to the similarity between species and

the use of traits only present at specific growth stages. *Pycnanthemum* can be misleading in its first year, growing low with sprawling shoots. However, in the second year, plants in the ground will double in height and develop erect shoots. The flowering of *Pycnanthemum* also intensifies during the second year, according to Dr. Svoboda V. Pennisi (personal communication).

The public could serve as a great ally in conserving pollinators. Previous studies have not attempted to breed *Pycnanthemum* for the landscape, though *Pycnanthemum* is a native pollinator-attractive plant (Harris et al., 2022; Porter, 2010) with appealing characteristics. Introducing *Pycnanthemum* to more urban and suburban landscapes could mitigate pollinator decline caused by habitat fragmentation. Urbanization reduces the floral resources available to pollinators, especially the native plants in an area. Losing native pollinator plants limits specialist pollinator populations (Braman & Griffin, 2022). Specialists may rely on native plants to obtain resources or shelter; therefore, removing native plants limits the resources available to specialists. One study observed pollinator visitation to native and exotic plants in urban green spaces (Zaninotto et al., 2023). The study found that non-native plant species provided significantly more floral area than native species during late summer (Zaninotto et al., 2023). However, pollinators preferred native species significantly more when floral resources were available (Zaninotto et al., 2023). The greater diversity of pollinator species visiting native plants earlier in the season could explain the higher attractiveness of natives compared to non-native species (Zaninotto et al., 2023). Specialists may have been attracted to the native species, while generalists were attracted to both. A greater variety of species visiting natives would result in more pollinators visiting the natives overall because the exotic species attract fewer specialized pollinators. Zaninotto et al. (2023) demonstrate the imbalance between plants visited by specialized pollinations when discussing the 40.2% of pollinators that visited only native plants,

while 12.3% of pollinators visited only exotic plants. Native plants enhance native pollinator and beneficial insect abundance, serving as food and overwintering resources (Porter, 2010). Using native plants, such as *Pycnanthemum*, as additional resources in landscapes enhances the pollinator supportiveness of urban spaces.

Despite *Pycnanthemum*'s potential, previous studies have not attempted to cultivate the genus. The native genus attracts abundant pollinators, possibly supporting specialists and generalist populations. *Pycnanthemum* has characteristics that appeal to consumers, including beautiful flowers, unique leaf coloring, and a pleasant fragrance. The attractive characteristics of the native would make it a great addition to the market. The genus also has a plethora of variability between species that could lend itself to a breeding program. Cultivating *Pycnanthemum* for the landscape would encourage greater use of *Pycnanthemum* and provide more resources for pollinators. Breeding *Pycnanthemum* for the landscape should center on characteristics related to aesthetics and maintenance. Landscape characteristics include low maintenance, attractiveness, and consistency. Consumers usually prefer compact and branched plants with many large flowers. Alternatively, breeding for pollinators must improve resource availability and attractiveness. Cultivating *Pycnanthemum* could offer the public a desirable plant for the landscape that also provides support for pollinators.

Production of *Pycnanthemum*

Plant production involves best practices for growing and caring for plants, aiming to grow plants quickly and efficiently. Improving efficiency in the greenhouse and field makes plants, especially new introductions, more accessible to breeding programs and nursery operations. Rooting hormones and plant growth regulators (PGR) are commonly used by growers to improve plant success. Rooting hormones are liquids or powders applied to cuttings

by dipping the propagules into the hormone. The purpose of rooting hormones is to expedite rooting and increase the survival of propagules. Plant growth regulators are mainly used to slow growth and shorten internodes, making plants more compact. Plant growth regulators are applied through drenches or foliar sprays. Determining production procedures for *Pycnanthemum* would make the genus easier for breeders and growers to integrate into their operations.

Propagation

A common rooting hormone is Indole-3-butyric acid (IBA); however, the ideal concentration varies across plants. IBA is available as a liquid or powder dip. After applying the rooting hormone to plants, IBA is transported into the peroxisome within cells and converted into IAA (indole-3-acetic acid), the active form of auxin (Frick & Strader, 2018). IBA encourages lateral root branching, adventitious rooting, and elongation of root hairs (Frick & Strader, 2018), which makes it ideal for propagation. Vegetative cuttings rely on root growth to supply nutrients and water for shoot growth; therefore, IBA can significantly affect the success of cuttings. However, previous studies have not documented the ideal IBA concentration or application method for *Pycnanthemum* cuttings.

The plant species and IBA dosage are factors that influence the success of propagation. Pêgo et al. (2019) compared the rooting of *Streptosolen jamesonii* cuttings when treated with 500, 1000, 2000, and 3000 mg/L of IBA and planted in different substrates. The study dipped propagules in each solution for 1 minute and compared survival and rooting to determine which IBA concentration was ideal for *S. jamesonii* cuttings (Pêgo et al., 2019). Researchers found that the survival or rooting percentage among treatments did not differ and reported around 95% survival and rooting (Pêgo et al., 2019). However, the number of roots per cutting peaked at around 1500 mg/L across substrates (Pêgo et al., 2019). On the other hand, Rehana et al. (2020)

compared *Crossandra infundibuliformis* cuttings when treated with 1000, 2000, and 3000 mg/L. The researchers dipped cuttings in each IBA solution for 10 seconds and later examined them for survival and rooting (Rehana et al., 2020). Rehana et al. (2020) found that 3000 mg/L was an ideal concentration, with 86.25% rooting, 100% survival, and 17.75 roots per cutting (the highest number of roots across treatments).

The two previous studies demonstrate that survival and rooting percentages cannot be directly compared between genera. *S. jamesonii* survival and rooting were not significantly different between 500 or 3000 mg/L treatments (Pêgo et al., 2019); however, maximum survival and rooting of *C. infundibuliformis* occurred at 3000 mg/L (Rehana et al., 2020). Survival and rooting serve as parameters for successful propagation, while the number of roots per cutting is associated with the speed of propagule growth. Cuttings with more developed root systems are likely to grow faster, improving the efficiency of the plant's production. The separate plants studied above also had individual optimal IBA concentrations for the number of roots per cutting. The number of roots per cutting of *S. jamesonii* was more abundant at 1500 mg/L treatments than at 3000 mg/L treatments (Pêgo et al., 2019), despite the greater number of roots per cutting in *C. infundibuliformis* occurring at 3000 mg/L (Rehana et al., 2020). The plants exhibited different responses to similar IBA treatments; therefore, ideal IBA concentrations must be determined for individual genera to improve production efficiency.

Increasing the concentrations of IBA applied to plants improves rooting, although a concentration that is too high can decrease rooting (Carpenter & Cornell, 1992). Tien et al. (2020) examined the response of *Solanum procumbens* to different IBA concentrations. The researchers treated *S. procumbens* cuttings with 500, 1000, 1500, and 2000 mg/L of IBA, dipped in solution for 15 minutes (Tien et al., 2020). Tien et al. (2020) found that cuttings treated with

500 mg/L of IBA had the highest sprout formation (92.34%), root number (32.25), and root length (6.8 cm). The propagules treated with 1000 mg/L, the next highest concentration, caused a reduction in sprouting (75.05%), root number (26.44), and root length (5.23 cm) (Tien et al., 2020). The higher IBA concentrations resulted in even lower sprouting and rooting (Tien et al., 2020). The efficacy of IBA treatments decreases when cuttings are treated with excess amounts of IBA. When the concentration of IBA exceeds the optimal dose, fewer propagules survive, and the cuttings may be weaker.

Previous studies have not evaluated propagation procedures for *Pycnanthemum*. Determining optimal IBA doses allows for more efficient propagation, which is especially important for plant introduction. Propagation success suffers when IBA concentrations are not ideal, particularly when the concentrations are higher than the optimal dose. When IBA concentrations are lower or higher than the ideal concentration, fewer cuttings survive, and those that do are not in peak condition, which wastes plant material and labor. IBA concentrations above optimal doses also waste additional resources by using more product than required. The ideal concentration of IBA differs between genera; therefore, establishing the optimal amount of IBA for each *Pycnanthemum* species will ensure that more propagules are successful during cultivation and resources are used efficiently.

Regulating Growth

Paclobutrazol (PP333; (2RS, 3RS)-1-(4-chlorophenyl)- 4, 4-dimethyl-2-(1H-1, 2, 4-trizol-1-yl)-pentan-3-ol) is the active component in certain commercial plant growth regulators, such as Bonzi®. Paclobutrazol inhibits the biosynthesis of gibberellin and, therefore, decreases cell elongation (Kurniawati et al., 2023). Reducing cell elongation affects internode length, which limits plant height without significantly affecting tissue accumulation. The inhibition of

cell elongation rather than cell division does not directly inhibit the growth of plants. Instead, restricting cell elongation constricts growth into a smaller area, which is desirable to consumers. Paclobutrazol can also decrease the chlorophyll content of plants, as seen in *Ocimum sanctum* (Divya Nair et al., 2009), encouraging plant growth and deepening the green color of plants.

The results of PGR applications depend on application methods, plant species, and the concentration of PGR applied. No record of paclobutrazol applications on *Pycnanthemum* species exists in the literature. However, previous studies have examined the efficacy of different application methods on other plants. Bañón et al. (2002) compared plant growth of *Dianthus caryophyllus* when sprayed and drenched with various concentrations of paclobutrazol. The researchers sprayed plants with 0.16, 0.35, 0.51, and 0.65 mg a.i./pot or drenched plants with 0.45, 0.7, 0.9, and 1.12 mg a.i./pot (Bañón et al., 2002). Spraying plants with 0.51 and 0.65 mg a.i./pot resulted in plant heights of 13.78 and 11.94 cm, respectively, while plants drenched with 0.45 and 0.7 mg a.i./pot grew to 10.49 and 9.28 cm, respectively (Bañón et al., 2002). The higher concentrations of spray applications produced taller plants than the lowest drench application, suggesting drenches are more effective in reducing plant height than sprays. Bañón et al. (2002) determined that paclobutrazol drenches provided a 51.9 – 64.3% reduction in plant height, while spray treatments decreased height by 9.2 – 45.2%. The experiment demonstrated that drenches could achieve more compact plants with lower concentrations than sprays, indicating drenches are more economical.

The mechanism for paclobutrazol uptake may explain the greater effectiveness of drenches compared to sprays. Paclobutrazol mainly moves up through the xylem with little mobility within the phloem (Desta & Amare, 2021). Ribeiro et al. (2011) explain that paclobutrazol has low solubility in water, which restricts the movement of paclobutrazol in the

phloem. Spray applications result in more localized effects due to the immobility of paclobutrazol in the phloem. On the other hand, drenches are more uniform because the application method takes advantage of the upward movement of the xylem (Early & Martin, 1988), allowing for more even distribution. Lower-concentration drenches could be more effective due to the uniformity and efficiency of xylem transport. Spraying plants with paclobutrazol would require higher concentrations to reach the same distribution of drenches. Therefore, drench applications are more effective than foliar sprays of paclobutrazol.

Similar to IBA treatments, plants have an optimal PGR concentration. PGR concentrations can affect plant traits beyond vegetative growth. Paclobutrazol limits plant height and can impact flowering, potentially damaging the plant or resulting in phenotypes that no longer appeal to consumers. Dasoju et al. (1998) compared vegetative growth and flowering characteristics of *Helianthus annuus* following various paclobutrazol treatments by drenching plants with 2, 4, 8, 16, or 32 mg a.i./L (Dasoju et al., 1998). Dasoju et al. (1998) report that plant height and flower diameter decreased as PGR concentrations increased (Dasoju et al., 1998). Shorter, more compact plants are valuable to consumers; however, flowering is equally desirable. Different PGR concentrations can improve one aspect of plants while reducing another. Production procedures must balance vegetative and reproductive traits to attain the highest quality plants. The plant traits growers improve will depend on the growers' target market. Exploring the effects of various concentrations and application methods for each genus will ensure that growers can optimize procedures for their goals.

Paclobutrazol is also capable of promoting drought tolerance in plants under water stress. Drought tolerance is conferred to plants by increasing relative water content, enhancing antioxidative enzyme activity, and stabilizing cell structures (Jungklang et al., 2017). Hu et al.

(2017) demonstrate the ability of paclobutrazol to increase the relative water content of *Myrica rubra*, suggesting the improvement is due to an increase in abscisic acid (ABA). ABA is a plant hormone that promotes stomatal closure; therefore, increasing concentrations of ABA reduces stomate aperture and reduces water lost to evapotranspiration (Desta & Amare, 2021). An increase in the peroxidase activity following paclobutrazol treatments also helps plants tolerate drought stress by reducing reactive oxygen species (ROS) (Fan et al., 2020). Bañón et al. (2023) observed a reduction in non-photochemical quenching after paclobutrazol treatment in *Salvia officinalis*, which indicates ROS levels were lower in plants treated with PGR. Proline is an amino acid that regulates osmosis within cells under environmental stress (Matysik et al., 2002). The proline concentration in *Daucus carota* increased after paclobutrazol treatment, which maintains the intracellular structure, stabilizes macromolecules, and preserves cell turgor (Gopi et al., 2007). Paclobutrazol induces drought tolerance by influencing hormone levels and enzyme activity to reduce cell damage.

The Genetics of *Pycnanthemum*

Previous studies have not attempted to breed *Pycnanthemum* for commercial use. However, past crosses have revealed genetic information. The genome sizes of *Pycnanthemum* species, which are not yet documented, could prove helpful when hybridizing. Determining the genome size would help develop breeding programs and cultivate a deeper understanding of the genus.

The Breeding of *Pycnanthemum*

Breeding programs rely on genetic variation to produce enhanced plants. Interspecific hybridization is an effective method of introducing different genes into a population. However, barriers to interspecific hybridization limit the exchange of genes between species. For example,

embryo abortion, unreceptive stigma (Kuligowska et al., 2015), or geographical distance (Chambers & Chambers, 1971) can prevent crossing. *Pycnanthemum* hybrids are rare due to distance and genetics. Even in regions where the range of different species may overlap, populations are scattered, limiting cross-pollination opportunities. However, the discovery of an apparent hybrid of *P. virginianum* and *P. pilosum* (Chambers & Chambers, 1971) confirmed that natural interspecific hybridization was possible within the genus.

After discovering natural hybridization, Chambers and Chambers (1971) and Chambers (1993) conducted studies to evaluate the compatibility between parents by artificially crossing species. First, pollen staining using cotton-blue dissolved in lactophenol was used to ascertain the viability of the progeny's pollen. Then, the compatibility of parents was inferred based on the viability of each progeny's pollen with high stainability, suggesting viable pollen and compatible parents. The studies determined *P. albescens* and *P. loomisii* crossed readily, producing flowering F1 progeny with high pollen stainability (Chambers, 1993; Chambers & Chambers, 1971). The studies also found *P. pycnanthemoides* produced flowering progeny with high stainability when crossed with *P. incanum* and *P. beadlei* (Chambers, 1993; Chambers & Chambers, 1971). Previous studies establishing parental compatibility can inform future breeding efforts and improve the efficiency of the breeding program.

The Genome Size of *Pycnanthemum*

The genome sizes of *Pycnanthemum* species, which are not yet documented, could prove helpful when hybridizing and identifying species. Characterizing the genome of *Pycnanthemum* and determining the genome size would help develop breeding programs and cultivate a deeper understanding of the genus. Genome size is an additional tool to reveal which species are most likely to hybridize successfully. Previous genomic studies have found that some genera have

genome sizes that vary between and within species, independent of ploidy levels (Sliwinska et al., 2022). Similar genome sizes should experience higher rates of successful hybridization, which can help guide breeding decisions. Morphological traits can also be difficult to distinguish within the *Pycnanthemum* genus; therefore, genome size could assist with species identification.

Flow cytometry is commonly used to calculate the genome size of plant species. Genome sizes can be calculated by comparing the genome of *Pycnanthemum* to a standard plant with a known genome size. The flow cytometer draws stained cells into a small tube, suspending them in a string of single cells. Then, a laser or UV light excites the dyed cell, releasing a signal. The flow cytometer converts these signals into points and plots each emittance on a graph of fluorescent intensity versus cell count at a given time (Ochatt, 2006). After plotting data, the genome size of the *Pycnanthemum* species can be calculated using the samples' fluorescence of and the standard's genome size. Genome sizes can be reported as 1C, 2C, and 1Cx values. 1C-values are the amount of DNA in a nonreplicated gametic cell, 2C-values are the total DNA in somatic cells, and 1Cx-values are the amount of DNA in a nonreplicated base chromosome (Sliwinska et al., 2022). 1Cx-values allow DNA content to be compared independent of ploidy level. The 1Cx-value is calculated by dividing the 2C-value by the ploidy level of the sample (Sliwinska et al., 2022).

Propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) are common stains used to dye cells during flow cytometry. PI binds to DNA by inserting itself between bases and stains all base pairs (Parris et al., 2010). Alternatively, DAPI specifically dyes AT base pairs (Parris et al., 2010). PI staining provides more accurate genome size estimations due to its effect on all four base pairs; however, DAPI uses a more straightforward procedure and is less susceptible to human error. Parris et al. (2010) conducted a study comparing the results of

genome size analysis using PI and DAPI stains. The study found no significant difference between the stains unless the base pair composition between the standard and sample differed greatly (Parris et al., 2010).

After determining each *Pycnanthemum* species' chromosome numbers and ploidy level, past studies have discovered that the genus has a mixture of ploidy levels. Table 1.1 provides the current understanding of ploidy level and chromosome number (Chambers & Hamer, 1992; Chambers, 1993; Chambers & Chambers, 1971).

Table 1.1. Table of chromosome numbers and proposed ploidy levels. The chromosome numbers were determined experimentally (Chambers, 1993; Chambers & Chambers, 1971), while ploidy levels were proposed by Chambers and Hamer (1992) (indicated by asterisks).

Genus *Pycnanthemum*	Chromosome Number (2n)	Ploidy Level*
P. albescens	38, 76	diploid, tetraploid
P. loomisii	38	diploid
P. curvipes	40	diploid
P. pycnanthemoides	72	tetraploid
P. incanum	76	tetraploid
P. clinopodioides	76	tetraploid
P. floridanum	78	tetraploid
P. flexuosum	36	diploid
P. setosum	76	tetraploid
P. muticum	40, 80, ca.108	diploid, tetraploid, pentaploid
P. tenuifolium	40, 80	diploid, tetraploid
P. pilosum	78	tetraploid
P. torreyi	80, 120	tetraploid, hexaploid
P. verticillatum	ca. 76-78	tetraploid
P. virginianum	80	tetraploid
P. montanum	40	diploid
P. beadlei	76	tetraploid
P. nudum	40	diploid
P. californicum	40	diploid

Gathering information on the genome size, chromosome number, and ploidy level of plant species provides a more comprehensive view of a plant's genomics. Examining the relationship between genome size and chromosome number or ploidy within a genus could help predict genetic information for species that have not been examined. Some genera have high correlations between genome size and ploidy level. Tamura et al. (1998) found the genome size and ploidy level of *Diospyros* species using flow cytometry. After establishing genome size, the researchers calculated the correlation between 2C genome size and ploidy level. The study found a strong positive linear correlation (R = 0.986) between ploidy level and the genome size, except for *D. montana*, a diploid with a genome size more similar to the genome size of a tetraploid species (Tamura et al., 1998). Alternately, Choi et al. (2020) found little correlation between the genome sizes of *Iris* species and their chromosome number. The chromosome number was determined using root tips, while flow cytometry revealed the genome sizes of each species (Choi et al., 2020). The study found a high variation of 1C genome sizes within chromosome number, suggesting little to no correlation between chromosome number and genome size (Choi et al., 2020). Examining the strength of the relationship between genome size and chromosome number or ploidy level informs predictions about genomic information when plant material is unavailable or current methods are not compatible with a species. Genetic information can be predicted with more certainty when the relationship between genome size and ploidy level or chromosome number is strong. Estimated genome sizes can guide hybridization or classification decisions and make breeding programs more efficient.

Inducing Genetic Variability to Increase Plant Diversity

Hybridization is one method of introducing variation into a breeding program. Crossing plants with different traits allows progeny with new phenotypes. Selfing plants also reveals

naturally available variability within a genus. Mutagenesis offers additional variability within breeding programs, providing the opportunity for new traits. Qualitative and quantitative traits can be altered due to mutagenic treatment, possibly introducing various unique characteristics into the breeding population. Studies have yet to introduce mutagenesis into a *Pycnanthemum* breeding program.

Mutagen treatments can cause silent changes (resulting in no visible modification), lethal mutation, or altered phenotypes. The correct dosage of mutagens must be applied to phenotypically modify plants and increase the variability within a breeding population. The LD_{50} is the dose at which 50% of the population survives and is most likely mutated (Raina et al., 2018). Thus, the interaction of dosage and exposure time can affect mortality and the successful mutation of plant material. Optimizing the dose of mutagens requires extensive testing for each plant species because the LD_{50} varies for each species and mutagen. Determining the LD_{50} provides researchers with the ideal dose of a mutagen for mutated phenotypes, which breeders can use to introduce genetic variability into breeding programs.

Ethyl methanesulfonate (EMS) is a chemical mutagen that mutates genes by substituting nucleotides in the genome, mainly converting guanine-cytosine pairs to adenine-thymine (Yan et al., 2021). The results of previous EMS treatment studies have ranged from mutations conferring variegated vegetation to mutated flower characteristics (Siddique et al., 2020). Siddique et al. (2020) treated *Capsicum annuum* seeds with a 1.3% EMS solution for 12 hours and examined the M_1 and M_2 populations. The M_1 population exhibited potentially desirable phenotypes such as altered plant height and foliage color (Siddique et al., 2020). Of the plants treated, 1.12% were taller than controls, 0.51% were shorter than control plants, and 0.61% developed albino leaves (Siddique et al., 2020). Mutations tend to be recessive, resulting in mainly silent mutations for

the first generation of treated plants (M_1 population). Therefore, visible mutations in the M_1 generation indicate that mutations in the M_2 population are highly likely. Within the M_2 population, 30.5% of plants had mutations related to plant growth, including height, stem development, and branching (Siddique et al., 2020). Siddique et al. (2020) found that 50.2% of plants in the M_2 population had mutations associated with leaf characteristics such as leaf color, texture, and shape. Among the second generation of mutated plant material, 12.3% of plants exhibited mutations related to flowering traits, including anther color, ovary size, and AGAMOUS mutations (Siddique et al., 2020). AGAMOUS mutations replace reproductive organs with petals, resulting in fuller flowers but limited reproductive viability. EMS is an effective mutagen that can introduce a variety of phenotypes into plant populations. The diversity of mutations in the previous study shows that EMS-induced mutagenesis affects many plant traits. EMS can produce characteristics that do not exist within the gene pool of the genus and would, therefore, be impossible to introduce through traditional breeding methods.

Mutagenesis induced by irradiation is another method for introducing variability. Irradiation has been studied on various ornamental and agricultural plants aside from *Pycnanthemum*. Gamma irradiation, specifically, mutates DNA through a variety of mechanisms. Exposure to gamma rays can change protein conformations, oxidize amino acids, destroy covalent bonds, and produce free radicals (Piri et al., 2011), affecting DNA and gene expression. Various plant structures can be treated with irradiation, including seeds, bulbs, cuttings, rhizomes, and seedlings (Datta, 2009).

The effects of irradiation on plants differ between genera and are dose-dependent. Oates et al. (2013) treated *Rudbeckia subtomentosa* with 0, 5, 10, 20, and 40 Gy gamma irradiation and examined the vegetative and reproductive characteristics of the M_1 populations. *Rudbeckia*

subtomentosa plants treated with gamma irradiation exhibited a decrease in plant height, flower number, flower size, and pollen viability (Oates et al., 2013). *Rudbeckia subtomentosa* displayed a direct and fairly linear relationship between plant characteristics and irradiation dosage. Alternatively, Shala (2019) found a more parabolic relationship between irradiation and plant traits. Shala (2019) treated *Ocimum basilicum* with 0, 5, 10, 15, 20, 25, and 30 kR of gamma irradiation and examined the M_1 populations. Shala (2019) found that plant height also decreased at the higher irradiation doses; however, branching increased in the plants treated with 10 kR. Additionally, phenotypic variation and unique characteristics increased in *Pavonia hastata* when plants underwent 1500 Gy (Yue & Ruter, 2020). *Pavonia* plants exhibited variable sizes, novel vegetative colors, and larger flowers compared to the control (Yue & Ruter, 2020). Irradiation does not affect all plants linearly, and treated plants' phenotypes differ across species. Some plants experience a steady decrease in height or branching from irradiation. Meanwhile, other species increase in height or branching when treated with irradiation. Therefore, the effect of irradiation must be examined for each species individually. Determining the optimal irradiation dosage also depends on the goals of a breeding program.

Breeding for Consumers and Pollinators

Breeding plants for consumers requires breeders to have specific goals in their breeding program. However, the attributes that appeal to consumers may not support pollinators. For example, some plants are bred for compactness, more petals, altered floral structure, and different flower colors or patterns. Owen and Bradshaw (2011) examined how mutating floral characteristics affected the visitation of bumble bees (*Bombus impatiens*). *Mimulus lewisii* seeds were soaked in EMS solution and self-pollinated to develop an M_2 population (Owen & Bradshaw, 2011). The researchers observed mutants for *Bombus impatiens* visitation, noting the

visit as “successful” if the insect came in contact with the anther and pistil or “attempted” if the bee was unable to come into contact with the flower’s reproductive organs (Owen & Bradshaw, 2011). Owen and Bradshaw (2011) found that mutants lacking their lower petals experienced 71% fewer visitations than controls due to the inability of bumble bees to gain enough structural support to enter the flowers. Although changing the form of flowers could make plants more attractive to consumers, the floral structure of some flowers serves a purpose for pollinators. Altering the floral shape within a breeding program could restrict pollinators from resources.

Owen and Bradshaw (2011) also found that 45% more bumble bees entered flowers upside down on mutants lacking nectar guides than wild-type plants. A separate mutant with a lighter petal color and less distinct patterning also experienced a decrease in visitation compared to the control (Owen & Bradshaw, 2011). Nectar guides are markings on the flower that direct pollinators to nectar and are only visible under ultraviolet light. Bees view flowers differently than humans. As a result, our visible light spectrum may hide significant changes in nectar guide patterning. If the visible coloring of a flower is modified, the non-visible color may also change, which could limit the ability of pollinators to access resources. The horticultural industry aims to cultivate plants with various colors and patterns; however, breeding programs must consider the effect of changing the coloring of flowers on pollinator resource localization.

Breeding programs rely on changing the genetics of plants to achieve a new and more appealing aesthetic. However, altering the genetics of plants could also affect pollinator attractiveness and resource accessibility. Examining the pollinator-attractiveness of parent plants would allow for comparisons of pollinator support between future cultivars and parents. Studies comparing pollinator attractiveness could help determine whether cultivars support greater or fewer pollinators than parent plants. No studies have compared wild-type *Pycnanthemum* to

Pycnanthemum cultivars, potentially due to a limited number of cultivated varieties. A few studies have compared pollinator attractiveness between *Pycnanthemum* and other genera species. However, the literature lacks research on pollinator attractiveness compared between *Pycnanthemum* species. Research on parent plants would allow the industry to label pollinator plants more confidently and ensure new landscape plants support pollinator populations.

Comparing Pollinator-attractiveness of Cultivars and Wild-type Plants

Encouraging public participation in pollinator conservation by marketing pollinator plants to the public requires pollinator plants to appeal to consumers. Therefore, plants must be cultivated for their consumer-friendly traits and pollinator support. Cultivars have different growth habits, vegetative characteristics, and reproductive traits than parent plants. For example, cultivars are usually more compact than the wild type and come in various colors and patterns. The issue, however, lies in whether the cultivars will be as attractive to pollinators and provide similar resources as the parent plants.

Ricker et al. (2019) compared the number of pollinators visiting different shrubs versus the cultivar of the species. The study observed *Aronia melanocarpa*, *Clethra alnifolia*, *Dasiphora fruticosa*, *Hydrangea arborescens*, *Kalmia latifolia*, *Physocarpus opulifolius*, and cultivars of each plant, categorizing visitors as *Apis mellifera*, *Bombus* spp., *Andrenidae*, *Halictidae*, *Megachilidae*, other bees, Lepidoptera, *Syrphidae*, other flies, wasps, Coleoptera, and other insects (Ricker et al., 2019). The study found that more *Andrenidae* visited *A. melanocarpa* than the cultivars, potentially due to the taller height of the wild-type (Ricker et al., 2019), which could account for the difference in *Andrenidae* visitations. Many butterflies also visit taller, more visible plants in a landscape (Baker et al., 2020). A more compact habit, which generally appeals to consumers, can make plants less visible to pollinators and reduce visitation.

Flower color is another important consideration within breeding programs. Many companies prefer to promote multiple plants with similar growth but different colors as a collection when releasing new cultivars. However, as discussed in the study by Owen and Bradshaw (2011), color can be integral in guiding pollinators to resources and attracting them to plants. *Dasiphora fruticosa* has yellow flowers, while *D. fruticose* 'Pink Beauty' has pink flowers. *Dasiphora fruticose* was visited by more *Bombus* spp. and *Megachilidae* pollinators than its cultivar, potentially due to differences in flower color (Ricker et al., 2019). Unlike *D. fruticose*, *C. alnifolia* attracted a similar amount of pollinators as its cultivars despite different flower colors (Ricker et al., 2019). The effect of flower color varied between plant genera. Therefore, studies must determine the impact of flower color on pollinator visitation for each genus.

One goal of plant breeders is to increase flower size and coverage. *Hydrangea arborescens* has showy, sterile flowers with small, fertile flowers. Therefore, cultivating *H. arborescens* involves increasing the number of showy flowers, which decreases the fertile flowers. Ricker et al. (2019) found that *Bombus* spp. visited *H. arborescens* three times as often as the cultivars. The significant difference in visitation could be due to *Bombus* pp.'s preference for flowers with more resources (Ricker et al., 2019). *Hydrangea arborescens* had 99% fertile flowers, while cultivars had 42% fertile flowers (Ricker et al., 2019), suggesting the cultivar also had significantly fewer resources to provide pollinators. Sterility is commonly incorporated into breeding programs because plants can focus on vigor and flowering instead of fruit development. However, sterility decreases pollinator resources, which could limit the potential of cultivars as pollinator plants.

Comparing the pollinator attractiveness of cultivars against wild types ensures new plant introductions support pollinators to the same extent as parent plants. Pollinators rely on some wild-type traits to locate and provide resources. Cultivated plants can alter the cues pollinators rely on, decreasing the value of those cultivars as pollinator plants. However, some cultivars are more attractive to pollinators than the wild type. Ricker et al. (2019) observed significantly more flies in the *Syrphidae* family visiting *Physocarpus opulifolius* cultivars than the wild-type. *Physocarpus opulifolius* has green foliage with white flowers, while *P. opulifolius* 'Monlo' has purple foliage with white flowers. The study suggests that more flies visited the cultivar because syrphid flies prefer yellow and white flowers, which stood out more against darker foliage. The cultivar and wild type could also have different olfactory cues and potentially affect pollinator visitation (Ricker et al., 2019). Cultivating a plant changes the genetic expression of the cultivar and can alter the attractiveness of the plants to pollinator populations. Understanding how breeding influences pollinator visitation will help validate future pollinator plant labels.

Pollinator-attractiveness Among Plant Species

Comparing pollinator attractiveness within a genus can also help improve the pollinator support provided by cultivars. Plant species may differ in the abundance and diversity of pollinators they attract, which could help guide a breeding program. Browning et al. (2023) compared pollinator visitation between different *Tagetes*, *Portulaca*, and *Bidens* cultivars. There was a significant difference between many of the cultivars of each genus independent of species within the *Tagetes* and *Bidens* (Browning et al., 2023). However, species affected visitation to *Portulaca*, with significantly more pollinator visitation to *Portulaca oleracea* than *Portulaca grandiflora* (Browning et al., 2023). Differences in nectar guides, volatile compounds, or floral morphology between the species may account for the contrast in pollinator attractiveness.

Portulaca oleracea may look different under UV light than *P. grandiflora*, affecting the accessibility of resources to pollinators. The chemicals emitted from flowers also act as a cue to some pollinators. A greater abundance of pollinators may have visited *Portulaca oleracea* due to the stronger or more appealing aroma of the species compared to *P. grandiflora*. *Portulaca grandiflora* also has more petals, which could alter the appearance of nectar guides or make it more difficult for pollinators to reach resources. Differences in pollinator attractiveness exist between the species of some genera. Determining which genera have interspecific variation and which species are most attractive could inform a breeding program. Beginning a breeding program with a species that attracts the highest abundance of pollinators within its genus improves the potential pollinator attractiveness of the end product.

Encouraging Public Participation in Pollinator Conservation

The public could serve as a great ally in conserving pollinators suffering from the effects of habitat fragmentation in rural and urban areas. Breeding pollinator plants with landscape traits will encourage the implementation of the plants in urban landscapes, which will provide more resources to pollinator populations. Conserving pollinators by breeding pollinator plants requires plants to be accessible to growers, appealing to the consumer, and support pollinators throughout cultivation.

Growers must successfully produce new plants for the product to reach the market. Determining propagation and growth regulation procedures will assist production by providing growers with the tools to confidently and efficiently grow new pollinator plants. Consumers must also desire the plant to use the cultivar in their landscape. Pollinator plants must appeal to consumers to effectively impact pollinator conservation. Breeding pollinator plants for the landscape will garner interest in the plants and potentially pollinator conservation. Maintaining

pollinator support traits throughout breeding is also crucial to using pollinator plants to encourage conservation. Plants must balance consumer appeal and pollinator support, or risk becoming useless to pollinators. Validation of pollinator plants will ensure plants in the landscape support local pollinator populations.

Pycnanthemum is a pollinator plant with market potential, providing pollinator attractiveness with beautiful flowers, fragrance, and growth habit. The genus also offers the opportunity to introduce more diversity in landscapes by acting as an additional pollinator plant to those already on the market. Diversity is integral to supporting pollinators; adding pollinator-attractive plants to landscapes will ensure pollinators have access to resources. *Pycnanthemum* has untapped potential as a pollinator plant for the landscape. Exploring the genus will assist future breeding and production efforts to bring *Pycnanthemum* to the market as a beautiful plant the public can use to improve their landscape and participate in pollinator conservation.

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CHAPTER 2

COMPARING POLLINATOR SPECIES RICHNESS AND ABUNDANCE BETWEEN *PYCNANTHEMUM* SPECIES AND ACCESSIONS

[1]Swiantek, K. and J. Ruter. To be submitted to Frontiers in Sustainable Cities

Abstract

Pollinator populations are experiencing a global decline despite their role in the ecosystem, human health, and the economy. *Pycnanthemum* is a pollinator-attractive plant with a potential market value that could encourage the public to supplement pollinator resources in the landscape. Comparing the pollinator attractiveness of *Pycnanthemum* species and accessions allows breeders to select plants that maximize pollinator abundance and species richness. Pollinators were observed and captured while visiting three species of *Pycnanthemum* and five accessions (*P. flexuosum* (F), *P. virginianum* (V), and three accessions of *P. tenuifolium* (T1-T3)) to compare pollinator visitation. *Pycnanthemum flexuosum* consistently attracted an abundance of Lepidoptera, Diptera, honey bees (*Apis mellifera*), bumble bees (*Bombus spp.)*, carpenter bees (*Xylocopa spp.*), and small bees during observations. Plant V attracted the highest abundance of pollinators overall, with Apis mellifera (honey bees) accounting for over 50% of the pollinator visitation. Honey bees and *Xylocopa spp*. (carpenter bees) visited each species equally. Diptera (flies) were most attracted to Plants F, T2, T3, and V. Plants T3 and V attracted the highest abundance of wasps. Bombus spp. (bumble bees) visited Plants F and V more than other plants. Plant F attracted the greatest number of small bees and Lepidoptera (butterflies and moths). All plants attracted the same species richness of pollinators, ranging from visitation of at least 24 to 29 different pollinator species. Resource availability, plant morphology, and olfactory cues may have influenced pollinator preferences. Establishing which *Pycnanthemum* species attracted the highest abundance and species richness of pollinators will lay the foundation for conservation and cultivation.

Introduction

Pollinators are crucial to our society, influencing the ecosystem, human health, and the economy. Our environment relies on pollinators because they function as ecological bridges due to their impact on multiple links in the food chain. Pollinators support herbivores by encouraging plant reproduction (specifically pollinating 87.5% of angiosperms and an estimated 308,006 species) while acting as prey to predators (Ollerton, 2021). Pollinators are integral to multiple trophic levels; therefore, they impact the biodiversity and stability of ecosystems (Bailey, 2022; Porter, 2010). Herbivores rely on plant pollination to survive, while predators depend on pollinators to promote abundance in prey species. Pollinator diversity also influences ecosystem stability through functional redundancy, which protects ecosystems from sudden gaps in the ecosystem network (Bendel et al., 2019). Humans rely on pollinators to pollinate the crops we consume and feed to our livestock. Pollinators are responsible for pollinating 87 international commercial crops (Klein et al., 2007) and are valued at $235 – $577 billion globally for crop production (Ollerton, 2021). Pollinators also support the economy through the pest control services they provide. For instance, certain pollinators within Hymenoptera and Diptera provide additional pest control through predation and parasitism (Amarasekare, 2020). Losey and Vaughan (2006) determined the annual cost of pest control supplied by pollinators in the United States to be $4.5 billion. Additionally, the crop pollination provided by native bees in the United States was valued at an estimated $3.07 billion (Losey & Vaughan, 2006). The pest control and pollination services pollinators provide improve crop yields by reducing pest pressure and facilitating crop reproduction, which decreases the cost of inputs for agricultural operations and, therefore, lowers the cost of food.

Pollinator populations have been declining in recent years despite their importance in our society. The coverage of Colony Collapse Disorder in 2006 directed the pollinator decline discussion toward honey bee populations; however, other pollinator populations are also suffering, including monarch butterflies, bumble bees, and native bees (Bailey, 2022). Changes in land use, expansions of commercial agriculture, and loss of resources are the main factors negatively impacting pollinator survival (Potts et al., 2010). Habitat fragmentation caused by urbanization is one aspect of changing land use responsible for the declining pollinator populations (Rhodes, 2018). As cities expand and more land is used for human activity, the natural area shrinks. When human activity causes habitat fragmentation, pollinators become isolated from resources and other pollinator populations. Steffan-Dewenter et al. (2006) explain that insects in higher trophic levels are more vulnerable, though all pollinator populations are likely to decline due to inbreeding or unpredictable extinction events. Habitat fragmentation restricts the resources available to pollinators and alters their environment, making the insects more susceptible to suddenly harsh conditions. The limited resources within fragments force pollinators to search for resources further from their nesting areas and reduce foraging success (Khalil, 2021). Pollinator survival is significantly reduced in habitat fragments due to a lack of resources and isolation from other populations.

Introducing plants into the urban landscape could help mitigate the effects of habitat fragmentation by providing additional resources and reconnecting pollinator habitats. Menz et al. (2011) discuss the stepping-stone method of linking pollinator habitats, which involves using individual, small-scale plantings to connect isolated fragments. In urban settings, patches of pollinator plants could consist of trees along sidewalks, small flowerbeds, or plants hanging from windowsills. Native wildflowers offer a low-maintenance and attractive landscape for cities

(Bretzel et al., 2009). Using existing green space in cities to introduce pollinator plants would support pollinator populations. Generalist plant species should be prioritized when connecting pollinator habitats to provide consistent resources and stability over time (Zografou et al., 2020). Pollinators require an abundance of nectar and pollen from plants. Syrphid flies (*Syrphidae*), butterflies and moths (Lepidoptera), and bees consume pollen and nectar as adults, while other pollinators, including parasitic bees and certain solitary bees, rely on nectar (Khalil, 2021). The plants introduced into pollinator habitat must offer abundant and diverse resources to support pollinators. Resource availability is crucial to pollinator attractiveness, influencing pollinator behavior over plant diversity (Battle et al., 2021). Habitat restoration can be introduced in small-scale gardens or parks; however, the plants must support a range of pollinators and provide ample resources.

Entomologists identify *Pycnanthemum* as a pollinator-attractive plant. In 2020, MacLeod et al. studied the flower preferences of crop-pollinating and rare bees. After observation and pollinator netting, MacLeod et al. (2020) found that *P. tenuifolium* attracted quadruple the crop-pollinating bees as *Asclepias tuberosa* and twice the number of rare bees compared to *Solidago rigida*. *Asclepias tuberosa* and *S. rigida* attracted the second-highest abundance of pollinators (MacLeod et al., 2020), indicating that *Pycnanthemum* attracted significantly more bees than all other plants in the study. Cadotte et al. (2017) found that the *Pycnanthemum* clade attracted the most pollinators. However, the study did not comment on comparisons between the species used during the experiment (*P. tenuifolium* and *P. virginianum*) (Cadotte et al., 2017). Previous studies have also demonstrated that *Pycnanthemum* attracts a diversity of pollinators. Porter (2010) reported *P. flexuosum* attracted the most beneficial arthropod families compared to seven other species. Similarly, *P. verticillatum var. pilosum* attracted over 19 families of pollinators

across orders when evaluating visitation before and after blooming (Harris et al., 2022). *Pycnanthemum* is a pollinator plant that attracts a diversity and abundance of pollinators. Therefore, *Pycnanthemum* could be introduced into the landscape to support pollinators suffering from habitat fragmentation.

Pycnanthemum (mountain mint) is a member of the *Lamiaceae* with a mint or thyme-like fragrance and tubular flowers characteristic of the family. These herbaceous perennials are native to North America and have white or purple flowers with purple speckling. *Pycnanthemum flexuosum* grows between 0.5 – 1.2 m tall, with flowers that are 2 – 4 cm wide (Chambers, 1961b), and is endemic to the southeastern United States, stretching up to Virginia and out to Mississippi (Weakley, 2020). *Pycnanthemum virginianum* is 0.6 – 1.1 m, with flowers that are 0.8 – 1.5 cm wide (Chambers, 1961b). The species grows north of Georgia and out to North Dakota (Weakley, 2020). The height of *P. tenuifolium* ranges from 0.4 – 1 m tall, and the species has the smallest flowers (0.5 – 1 cm wide) of those discussed (Chambers, 1961b). *Pycnanthemum tenuifolium* is native from the east coast out to Texas and north of Florida's panhandle (Weakley, 2020). All the species described prefer moist to wet conditions (Wofford, 1989).

Pycnanthemum has potential as a pollinator plant for the market. Cultivating *Pycnanthemum* for landscape use will make pollinator conservation more accessible to the public. Providing consumers with a pollinator plant they desire in their home and city landscapes will help reconnect fragmented pollinator habitats and introduce more resources. Determining which species are most pollinator-attractive is valuable to breeders implementing the genus in their programs. Beginning the breeding program with the most pollinator-attractive species will produce the best cultivar for pollinators. In some cases, breeding plants can reduce pollinator

attractiveness by affecting traits such as color (Owen & Bradshaw, 2011) and resource availability (Ricker et al., 2019). Determining foundational pollinator-attractive information ensures breeders can compare cultivars to parents. *Pycnanthemum* cultivars should maintain or improve pollinator attractiveness to be an effective pollinator plant in the landscape.

Materials and Methods

Plant Material and Plot Arrangement

P. flexuosum, *P. virginianum,* and three accessions of *P. tenuifolium* were requested from the USDA-ARS Western Regional Plant Introduction Station in Pullman, WA (Table 2.1). Seeds were sown into 7.62 (427 ml) CN-SP-40 Sheet Pots (Greenhouse Megastore, Danville, IL) with Pro-line C/B Growing Mix (Shady Dale, GA) in the greenhouse (day/night temperatures were 25°C /20 °C and 40% / 30% relative humidity). Once seedlings were established, they were transplanted in the greenhouse into 11.43 cm (956 mL) Kordlock Square Pots (The HC Companies, Twinsburg, GA) with Pro-line C/B Growing Mix and fertilized with 8 g of Osmocote Plus 15-9-12, 8-9-month (15-4-10 N-P-K) (ICL Specialty Fertilizers, Summerville, SC), along with a weekly liquid feed injected at 200 mg/L (Jack's Professional Water Soluble Fertilizer 20-10-20 Peat-Lite, JR Peters Inc., Allentown, PA) (20-4.4-16.6 N-P-K). Plants in the greenhouse received overhead watering.

Once plants were established in the 11.43 cm (956 mL) pots, they were transplanted into the ground at the University of Georgia's Durham Horticulture Farm (33.944507, -83.375774). The experimental plot was composed of Cecil sandy loam (USDA). The five accessions of *Pycnanthemum* were planted in a single row, spaced 1 meter apart. Plants were labeled as Plant F (*P. flexuosum,* W654808), T1 (*P. tenuifolium*, W657304), T2 (*P. tenuifolium*, W654752), T3 (*P. tenuifolium*, W654763), and V (*P. virginianum*, W657076). The experimental layout was ten

randomized complete blocks with one replicate of each accession per block. Plants were irrigated using 20 mm wide drip tape with emitters spaced 30 cm apart (Rivulis Irrigation Ltd., San Diego, CA). Plants were irrigated for 90 minutes four times a week for the first year and rain-fed during the second year. The plot was fertilized once during the first year with Imperial Supreme 16-4-8 Lawn Fertilizer (16-1.8-6.6 N-P-K) (Athens Seed Lawn & Garden, Watkinsville, GA).

Data Collection

Observations of each plant were taken biweekly, for two minutes between June – August 2022 and 2023, when all plants were in bloom. The average temperature and cumulative rainfall during the experiment are listed in Table 2.2 (University of Georgia College of Agricultural & Environmental Science, 2023). Observation and insect capture occurred between 10 A.M. and 3 P.M. (Larson et al., 2014). The order of plant observation was randomized each week. During pollinator observations, insect visitation was tallied as Lepidoptera, Diptera, honey bees (*Apis mellifera*), bumble bees (*Bombus spp.)*, carpenter bees (*Xylocopa spp.*), small bees, and wasps (Hymenoptera). Insects were tallied each time they landed on the plant and remained on the flowers. Pollinator visitation is reported as a percentage of total attractiveness over the collection period.

The capture of insects included netting and aspiration. The tops of plants were swept with nets six times for each plant. Then, small pollinators were vacuumed into a small container using an aspirator (Forestry Suppliers, Jackson, MS) for two minutes per plant. Captured insects were placed in a freezer and pinned for identification.

During the second year of growth, the height and number of stems were averaged across plants.

Statistical Analysis

A Poisson regression (RStudio, PBC, Version 1.4.1725, Boston, Massachusetts) was used for statistical analysis to determine if the plant accession or species significantly affected pollinator abundance and species richness (O'Hara & Kotze, 2010). The interaction between the year and pollinator visitation was significant, though the study focused on the total pollinator attractiveness. Therefore, the visitation of pollinators was averaged over the two years of data collection to compare the total pollinator abundance and species richness across plants over both years. A Tukey HSD post-hoc test (RStudio) was used to compare the visitation between plants. Significance was reported as $p \leq 0.05$.

Results

Plant F grew between 0.5 – 0.9 m with 64.3 stems on average, Plant T1 was 0.5 – 0.8 m tall and had 139.7 stems, and Plant T2 was 0.5 – 0.8 m with 89.7 stems. The height of Plants T3 and V ranged from 0.5 – 0.7 m with 192.7 stems and 0.9 – 1.1 m with 98.3 stems, respectively.

Plant V attracted the highest abundance of pollinators, with *Apis mellifera* (honey bees) accounting for over 50% of the pollinator visitation. Honey bees and *Xylocopa spp*. (carpenter bees) visited each species equally (Figure 2.1A-B). Diptera (flies) were most attracted to Plants F, T2, T3, and V (25, 21.23, 18.87, and 25.94% respectively) (Figure 2.1C). The abundance of flies attracted to Plant T3 was not different from Plant T1 (8.96%) (Figure 2.1C). Plants T3 and V attracted the highest abundance of wasps (30.21 and 29.76%, respectively), while wasps visited Plants F and T1 (12.23% and 10.82, respectively) the least (Figure 2.1D). Plants F and V were visited by more *Bombus spp.* (bumble bees) (38.15 and 35.26%, respectively) than all other plants (Figure 2.1E). Plant T1 was visited by significantly more bumble bees (11.56%) than Plant T2 (5.2%), though Plant T3 was not different from either (Figure 2.1E). Small bees visited

Plant F more often than all other plants in the study (52.28%) (Figure 2.1F). Lepidoptera (butterflies and moths) were attracted to Plant F more than all other plants in the study (30.68%) (Figure 2.1G). Plants T1 and T3 attracted a similar abundance of Lepidoptera (16.39% and 20.82%), though Plant T2 was visited by significantly fewer Lepidoptera (11.18%) (Figure 2.1G). All plants attracted the same species richness of pollinators, ranging from visitation of at least 24 to 29 different pollinator species (Table 2.3).

Discussion

Pollinator Attractiveness Among *Pycnanthemum* Species and Accessions

Among the species tested in this study, *Apis mellifera* are the most abundant pollinators attracted to *Pycnanthemum*, possibly due to the multiple hives existing onsite. Honey bees and *Xylocopa spp*. (carpenter bees) visited each species equally (Figure 2.1A – B). *Apis mellifera* are supergeneralists (Willmer, 2011a), suggesting the species do not have specific plant preferences and are opportunistic pollinators. Similarly, many carpenter bees are also generalists; however, they are larger-bodied pollinators that benefit from medium to large, sturdy flowers (Bin Farook et al., 2022; Willmer, 2011a). *Pycnanthemum* species have inflorescence composed of a multitude of flowers, closely bundled, which could provide an adequate platform for carpenter bees to land. Additionally, *Pycnanthemum* does not have traits that specifically repel pollinators, explaining the equal visitation in the study by the two generalist pollinators.

Diptera (flies) were equally attracted to each plant besides Plant T1 (Figure 2.1C). A combination of visual and olfactory signals attracts fly pollinators. Kastinger and Weber (2001) describe a study in which *Bombylius fuligino*, a member of the *Bombyliidae* (a family of Diptera pollinators), reacts to the volatile compounds of *Muscari neglectum* only when the concentration of the volatile compounds was perceivable by humans. Similarly, Shuttleworth and Johnson

(2010) demonstrate the role of volatiles in pollinator preference when comparing the attractiveness of *Eucomis* species with similar morphology, nectar composition, and flower color to fly pollinators (Shuttleworth & Johnson, 2010). The study found that flies visited flowers with higher concentrations of sulfur compounds in the scent chemistry (Shuttleworth & Johnson, 2010). The study demonstrates the possibility of related plants emitting different olfactory signals, resulting in variations in pollinator attractiveness. Carr and Hunter (1973) show the difference in the flavonoid profile between *Pycnanthemum* species, including *P. flexuosum, P. tenuifolium,* and *P. virginianum.* Therefore, Plants F, T2, T3, and V may be more attractive to fly pollinators due to a difference in the composition or concentration of the plant's volatile compounds. Of the *P. tenuifolium* in the study, Plant T2 was visited by more flies than Plant T1 (Figure 2.1C), while Plant T3 did not attract a different abundance of flies than the other *P. tenuifolium*. Carr and Hunter (1973) observed intraspecific variation in the chemical components of *P. tenuifolium* plants, which could affect pollinator visitations. Each accession of *P. tenuifolium* was morphologically similar, though differences in aromatics could account for the variable fly attraction across plants.

Wasps were most attracted to Plants T3 and V and visited Plants F and T1 the least (Figure 2.1D). Wasp Hymenoptera generally prefer dull flowers with easily accessible nectar (Kevan & Baker, 1983). Plants T3 and V have smaller flowers (Weakley, 2020) than Plant F, positioning nectar deposits closer to the flower opening and improving accessibility. Similar to flies, wasps use olfactory signals to locate flowers. In the same study mentioned previously, Shuttleworth and Johnson (2010) determined that wasps are attracted to *Eucomis* species with fewer sulfur compounds emitted from flowers. The attractiveness of the *Pycnanthemum* plants to wasp pollinators could be influenced by the different flavonoid profiles produced by each species

(Carr & Hunter, 1973). Wasp visitation also differed between each of the *P. tenuifolium* accessions (Figure 2.1D), which supports a volatile-related preference rather than a morphological difference. The *P. tenuifolium* accessions have similar flower size, leaf shape, and growth habits, though the olfactory cues emitted by *P. tenuifolium* differ within the species (Carr & Hunter, 1973). Therefore, wasps may be able to differentiate between the accessions due to a difference in volatile compounds.

Plants F and V attracted more *Bombus spp.* (bumble bees) than all other plants (Figure 2.1E). A previous study compared bumble bee visitation between flowers of different colors when one flower had a constant source of nectar and the other had variable nectar rewards (Real, 1981). Real (1981) found bumble bees visited flowers with consistent nectar resources compared to variable rewards, even disregarding flower color preferences to target flowers with more nectar constancy. The results indicate the pollinators learned which plants provided more resources. Plants F and V could have more consistent nectar rewards than other plants, explaining the attraction of bumble bees. In addition to distinguishing between different colored flowers, bumble bees discriminate against flowers based on predicted resource availability. In a study comparing damaged and undamaged flowers, bumble bees approached unblemished flowers more often, potentially due to the perceived rewards (Goulson et al., 2007). Goulson et al. (2007) suggest that damaged flowers often correlate with less nectar, and older flowers may have had their resources removed by previous visitors. The study demonstrates that bumble bees learn to focus foraging efforts on maximizing nectar collection. Kevan and Baker (1983) describe bumble bees visiting fewer *Oxytropis splendens* plants as the color of the banner petals dulls, demonstrating how bumble bees use color to focus foraging on newer flowers. *Pycnanthemum flexuosum* has whitened calyxes, which look like flower blooms from a distance.

Bumble bees may be mistaking the whitened calyxes as additional fresh resources and visiting Plant F more often. Plant T1 was visited by significantly more bumble bees than Plant T2, although Plant T3 was not different than either (Figure 2.1E). The unequal bumble bee visitation between *P. tenuifolium* accessions may be due to a difference in the nectar rewards offered by the plants. Plant T1 could be a more consistent nectar source than the other *P. tenuifolium* accessions in the study, which would encourage bumble bees to discriminate against riskier resources.

Small bees visited Plant F more often than all other plants in the study (Figure 2.1F). A previous study found that small bees preferred strawberry flowers with larger petals and intact stamen (Ashman et al., 2000). Larger petal areas and stamen presence provide pollinators with a more distinct visual in the landscape, which may assist pollinators with locating floral resources. Large petals contrast visually from vegetation either texturally or in regards to color (Ashman et al., 2000). Additionally, stamen fluoresce under UV light and may release olfactory cues that communicate pollen rewards, attracting pollen-consuming small bees (Ashman et al., 2000). Plant F has the largest flowers of the species in the study and protruding stamens (Chambers, 1961b), both of which encourage small bee visitation.

Lepidoptera (butterflies and moths) were attracted to Plant F more than all other plants in the study (Figure 2.1G). Butterflies land to feed (Kevan & Baker, 1983), which requires sturdy inflorescences large enough for the Lepidoptera. The inflorescences of *Pycnanthemum* is densely clustered flowers, providing a sturdy landing area. Butterflies and moths also prefer clustered, long, tubular flowers with protruding stigma and stamen (Willmer, 2011b), which can be accessed with their long proboscis (Kinoshita et al., 2017). Plant F has the largest individual flowers compared to the other species in the study, encouraging butterflies to visit Plant F more

than the other species. Similar to bumble bees, butterflies prefer new flowers with more nectar (Goulson et al., 2007) and adjust flower preferences to maximize nectar rewards. Kinoshita et al. (2017) found *Papilio xuthus* learned which flower color indicated greater reward after only eight days. Plant F may provide the greatest nectar rewards compared to other plants, which attracted a greater abundance of butterflies over the season. Butterflies also use color and volatile compounds to locate flowers. Color is mainly responsible for attracting *Pieris* and *Vanessa* butterflies to flowers from long distances; however, volatiles are a secondary cue butterflies use when the pollinators are in close proximity to flowers (Barragán-Fonseca et al., 2020). Butterflies prefer weak, fresh, and slightly sweet aromas with high concentrations of benzenoids, terpenoids, and green leaf volatiles (Kinoshita et al., 2017). Each species in the study is white, which suggests the preference for Plant F could be explained by a difference in volatile compounds (Carr & Hunter, 1973). Plant F may have experienced a greater abundance of butterfly visitation because the species released olfactory cues more attractive to butterflies. Flower degradation of *P. flexuosum* may also be more difficult for butterflies to determine due to the whitened calyxes that mimic blooms and hide flower decay. Plants T1 and T3 attracted a similar abundance of Lepidoptera, while Plant T2 was visited by significantly fewer Lepidoptera (Figure 2.1G). The *P. tenuifolium* accessions have similar floral morphology; therefore, a difference in the olfactory cues of each accession (Carr & Hunter, 1973) may be the cause of the variable butterfly visitation rates.

Pollinator Species Richness Among the *Pycnanthemum* Species and Accessions

The species richness did not differ between plants. *Pycnanthemum* is a member of *Lamiaceae*, which commonly requires insect pollination (Mačukanović-Jocić et al., 2011). Therefore, members of the *Lamiaceae* benefit from abundant nectar and similar traits aimed to

attract a variety of pollinators (Khalil et al., 2023). Alternatively, some species in the *Lamiaceae* have specialized floral morphology, such as anthers tucked towards the base of the corolla (Danforth et al., 2019). Pollinators that gather pollen for larvae, such as bees and *Masarinae* (pollen wasps), insert their head into the flower and vibrate to access pollen that is then deposited onto the insect (Khalil, 2021). Conversely, *Pycnanthemum* flowers have short corollas with protruding stamen, which provide easy access to pollen and nectar and make the genus more accessible to a larger variety of pollinators. The flower morphology of *Pycnanthemum* makes nectar and pollen readily available. Additionally, the olfactory cues of *Pycnanthemum* are mild, which does not exclude most pollinators. *Pycnanthemum* species also have hermaphrodite flowers, making the genus more appealing to many small bees and flies (Ashman et al., 2000). The variety of pollinators attracted to the *Pycnanthemum* plants used in the study does not differ significantly due to the robust pollinator-attractive traits of the genus.

Breeding *Pycnanthemum* for pollinators requires an initial assessment of the attractiveness of each species to determine which species is most attractive to pollinator groups. *Pycnanthemum flexuosum* attracted the greatest abundance of most pollinator categories. The species either attracted the most of a category or was on par with others (Figure 2.1), with the exception of wasps. *Pycnanthemum* has potential as a pollinator plant for the landscape; therefore, supporting pollinator abundance and species richness should be the main goal of the breeding program. Ensuring pollinators continue to gain support from *Pycnanthemum* species is integral to conservation efforts. The number of pollinators visiting a plant quantifies the pollinator support offered by the plant, while species richness indicates the breadth of the plant's impact on pollinator conservation. Future studies should compare plant traits, aromatic compounds, and pollinator resource availability to give context to the differing pollinator

preferences. Future studies should also consider the pollinator attractiveness of all 19 *Pycnanthemum* species. Breeding efforts may alter traits related to flower localization or resource abundance. Therefore, establishing the abundance and species richness of pollinators attracted to *Pycnanthemum* prior to cultivation lays the foundations for the expectations of the pollinator attractiveness of future cultivars.

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Tables and Figures

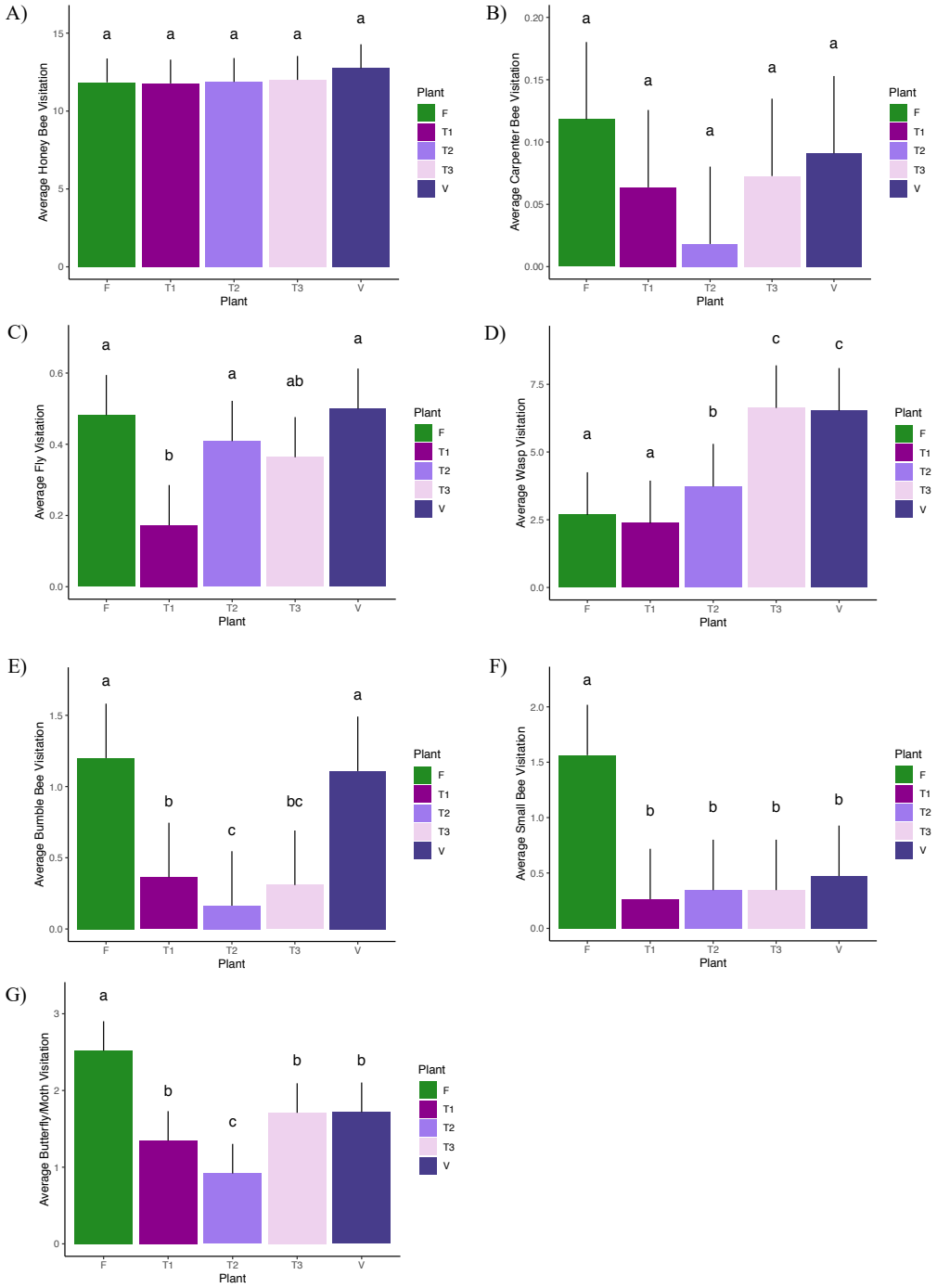

Figure 2.1. The mean abundance of pollinators observed visiting *Pycnanthemum*. The abundance of visitors was averaged over the collection period (biweekly observations between June – August during 2022 and 2023). The plants include *P. flexuosum* (F), *P. tenuifolium* (T1), *P. tenuifolium* (T2), *P. tenuifolium* (T3), and *P. virginianum*. The pollinator categories include (**A**) honey bees (*Apis mellifera*), (**B**) carpenter bees (*Xylocopa spp.*), (**C**) flies (Diptera), (**D**) wasp Hymenoptera, (**E**) bumble bees (*Bombus spp.*), (**F**) small bees, and (**G**) butterflies and moths (Lepidoptera).

Table 2.1. Information on the acquisition of germplasm.

Genus *Pycnanthemum*	Accession ID	Collection Data
P. flexuosum	W654808	Pullman, WA: USDA-ARS Western Regional Plant Introduction Station (seed); North Carolina
P. tenuifolium	W654752	Pullman, WA: USDA-ARS Western Regional Plant Introduction Station (seed); Maryland
P. tenuifolium	W654763	Pullman, WA: USDA-ARS Western Regional Plant Introduction Station (seed); North Carolina
P. tenuifolium	W657304	Pullman, WA: USDA-ARS Western Regional Plant Introduction Station (seed); New Jersey
P. virginianum	W657076	Pullman, WA: USDA-ARS Western Regional Plant Introduction Station (seed); Delaware

Table 2.2. The average temperature and cumulative rainfall. Averages obtained from the University of Georgia Weather Station in Watkinsville-Hort (http://www.georgiaweather.net).

Year	Month	Max Temperature (°C)	Min Temperature (°C)	Cumulative Rainfall (cm)
2022	June	28.8	17.8	18.01
	July	32.7	21.3	6.65
	August	32.1	21.1	18.11
2023	June	32.5	19.8	0.99
	July	32.1	21.8	13.56
	August	30.4	20.9	8.28

Table 2.3. The variety of pollinators captured on *Pycnanthemum*. Pollinator capture occurred biweekly between June – August during 2022 and 2023. *Pycnanthemum flexuosum* (F), *P. tenuifolium* (W657304; T1), *P. tenuifolium* (W654752; T2), *P. tenuifolium* (W654763; T3), and *P. virginianum*. All insects were identified to family, though pollinators were identified more specifically when possible.

Genus *Pycnanthemum*	Accession ID	Order				
		Wasp Hymenoptera	Bee Hymenoptera	Lepidoptera	Diptera	Coleoptera
P. flexuosum	W654808	*Ammophila spp.*	*Apis mellifera*	*Cisseps fulvicollis*	*Conopidae*	*Conotelus spp.*
		Braconidae	*Bombus spp.*	*Copaeodes minima*	*Syrphidae*	*Mordellidae*
		Eremnophila aureonotata	*Halictus confusus*	*Euptoica claudia*		
		Scolia spp.	*Halictus ligatus/poeyi*	*Hylephia phyleus*		
		Scolia dubia	*Halictus rubicundus*	*Junonia coenia*		
		Sphex pensylvanicus	*Lasioglossum spp.*	Moths		
			Megachile spp.	*Pyraloidea*		
			Melissodes spp.	*Strymon melinus*		
				Vanessa virginiensis		
P. tenuifolium	W657304	*Ammophila spp.*	*Apis mellifera*	*Atteva punctelle*	*Conopidae*	
		Bicrytes spp.	*Bombus spp.*	*Euptoica claudia*	*Syrphidae*	
		Chlorion aerarium	*Halictus confusus*	*Hylephia phyleus*		
		Chrysidinae spp.	*Halictus ligatus/poeyi*	*Junonia coenia*		
		Scolia spp.	*Halictus rubicundus*	Moths		
			Lasioglossum spp.	*Pyraloidea*		
			Megachile spp.	*Tortricidae sponganothis*		
			Triepeolus spp.	*Vanessa virginiensis*		
			Xylocopa spp.			

P. tenuifolium	W654752	*Ammophila spp.*	*Apis mellifera*	*Atteva punctelle*	*Bombyilidae villa*	*Mordellidae*
		Bicrytes spp.	*Calliopsis andreniformis*	*Euptoica claudia*	*Syrphidae*	
		Braconidae	*Halictus confusus*	*Hylephia phyleus*	*Palpada spp.*	
		Chalcidinae	*Halictus ligatus/poeyi*	*Junonia coenia*		
		Scolia spp.	*Lasioglossum spp.*	Moths		
		Scolia dubia	*Megachile spp.*	*Phycoides tharos*		
			Nomadinae	*Pyraloidea*		
			Xylocopa spp.	*Strymon melinus*		
P. tenuifolium	W654763	*Ammophila spp.*	*Apis mellifera*	*Euptoica claudia*	*Bombyilidae villa*	*Mordellidae*
		Bicrytes spp.	*Bombus spp.*	*Hylephia phyleus*	*Conopidae*	
		Euodynerus hidalgo	*Ceratina cockerelli*	*Junonia coenia*	*Syrphidae*	
		Podalonia spp.	*Halictus confusus*	Moths		
		Scolia spp.	*Halictus ligatus/poeyi*	*Pyraloidea*		
		Scolia dubia	*Halictus rubicundus*	*Strymon melinus*		
		Sphex ichneumoneus	*Lasioglossum spp.*	*Tinea pellionella*		
			Megachile spp.	*Vanessa virginiensis*		
			Nomadinae			
			Xylocopa spp.			
P. virginianum	W657076	*Bicrytes spp.*	*Apis mellifera*	*Atteva punctelle*	*Bombyilidae villa*	
		Euodynerus hidalgo	*Bombus spp.*	*Hylephia phyleus*	*Syrphidae*	
		Monobia quadridens	*Calliopsis andreniformis*	*Junonia coenia*		

Pachodynerus erynnis	*Coelioxys mitchelli*	Moths
Podalonia spp.	*Halictid parallelus*	*Pyraloidea*
Scolia spp.	*Halictus confusus*	*Strymon melinus*
Scolia dubia	*Halictus ligatus/poeyi*	
Sphex habenus	*Halictus rubicundus*	
Sphex ichneumoneus	*Lasioglossum spp.*	
Sphex pensylvanicus	*Megachile spp.*	

CHAPTER 3

THE EFFECTS OF PACLOBUTRAZOL ON THE VEGETATIVE AND REPRODUCTIVE TRAITS OF *PYCNANTHEMUM*

[1]Swiantek, K. and J. Ruter. To be submitted to HortScience

Abstract

Introducing pollinator plants into landscapes can mitigate pollinator decline caused by habitat fragmentation. *Pycnanthemum* would be considered a relatively new introduction to the market due to a lack of existing production procedures. Growers may be hesitant to work with the genus without an established production protocol; therefore, determining optimal plant growth regulator concentrations could make the plant more accessible. In the study, *P. virginianum* plants were drenched with 0, 0.5, 1, 2, 4, 8, and 16 mg a.i./pot of Bonzi®, while *P. flexuosum* were drenched with 0, 6, and 8 mg a.i./pot. The height, growth index, dry weight, density, and flower count were measured to compare growth between paclobutrazol concentrations. The height was effectively controlled in all treatments except *P. virginianum* treated with 0.5 mg a.i./pot. Flowering of *P. virginianum* increased at lower concentrations compared to higher concentrations. Treating *P. virginianum* with paclobutrazol decreased the shoot dry weight at higher concentrations. The growth index of *P. virginianum* increased at lower concentrations compared to high concentrations. *Pycnanthemum virginianum* treated with 8 mg a.i./pot increased plant density, though the density of plants treated with 16 mg a.i./pot were not different than the control. The height and flowering of *P. flexuosum* plants decreased when treated with paclobutrazol. Treating *P. flexuosum* with 6 mg a.i./pot also decreased shoot dry weight, although the dry weight increased when treated with 8 mg a.i./pot. Drenching *P. flexuosum* plants with paclobutrazol decreased the growth index and density compared to the control. Plant introductions can be daunting for growers if plants have no previous protocols for care. Providing information about *Pycnanthemum* production will enable more efficient production and faster distribution.

Introduction

Efficiency in the greenhouse and field is integral to the success of new plants. New introductions are exciting to consumers but can be daunting to a grower if no information exists regarding plant cultivation. Introducing production information and best practices for growing and caring for plants makes new genera accessible to cultivation efforts and growers. Morphology can be controlled through temperature and light, influencing flowering and height (Moe & Heins, 1990). However, chemical applications require less specialized equipment than other methods. Plant growth regulators (PGRs) are compounds that influence plant growth and development through means other than supplying nutrients (Rademacher, 2015). Improving plants for commercial production using PGRs has been achieved in a variety of ornamental plants, such as *Osteospermum*, *Dianthus,* and *Helianthus* (Bañón et al., 2002; Barrios & Ruter, 2019; Olsen & Andersen, 1995). Growers often use plant growth regulators to limit growth, which allow growers to space plants closer together and increase product quantity (Whipker & McCall, 2000). PGR treatments can also extend the saleable period of plants by increasing their shelf-life (Kurniawati et al., 2023). Plant growth may also be regulated to meet consumer demands for compact and floriferous plants. Previous studies have also effectively reduced the height of *Zinnia* and *Pelargonium* and increased *Iris* flowering following PGR treatments (Al-Khassawneh et al., 2006; Cox & Keever, 1988).

Paclobutrazol (PP333; (2RS, 3RS)-1-(4-chlorophenyl)- 4, 4-dimethyl-2-(1H-1, 2, 4-trizol-1-yl)-pentan-3-ol) is the active ingredient in certain PGRs, such as Bonzi® (Greensboro, North Carolina). Paclobutrazol limits plant growth by inhibiting gibberellin biosynthesis through a mechanism that prevents kaurene oxidation and the production of kaurenoic acid (Nagar et al., 2021). Gibberellin is responsible for cell elongation rather than cell division, which indicates

disrupting gibberellin biosynthesis restricts plant size (Jungklang et al., 2017). Paclobutrazol can decrease plant height and diameter (Dasoju et al., 1998). Paclobutrazol treatments can also influence the number of leaves, chlorophyll content, and activity of roots (Chandra & Roychoudhury, 2020). The chlorophyll content of *Ocimum sanctum* increased when plants were treated with paclobutrazol, which promotes photosynthetic processes and plant growth (Divya Nair et al., 2009). The ability of paclobutrazol to confer smaller size while encouraging growth characteristics produces more compact plants. Paclobutrazol also affects the reproductive stage of plant development. For example, Singh et al. (2008) describe an increase in flower abundance observed in *Salvia sclarea* following treatment with paclobutrazol.

Drought tolerance improves after paclobutrazol treatments. Plants treated with paclobutrazol require less water than control plants, as seen in *Abelmoschus esculentus* and *Salvia officinalis* (Bañón et al., 2023; Iqbal et al., 2020). Paclobutrazol induces stress tolerance through various mechanisms such as maintaining turgidity, enhancing antioxidative enzyme activity, and increasing the relative water content of plants (Jungklang et al., 2017). Gopi et al. (2007) found that proline levels increased with paclobutrazol concentrations in *Daucus carota*, which helps stabilize cells and macromolecules through osmotic regulation. The activity of reactive oxygen species was reduced in *Amorpha fruticose* and *Salvia officinalis* following paclobutrazol treatment (Bañón et al., 2023; Fan et al., 2020). Fan et al. (2020) reported an increase in peroxidase activity (an enzyme responsible for protecting plants against reactive oxygen species [ROS]) as the concentration of paclobutrazol increased. Similarly, Bañón et al. (2023) observed a decrease in non-photochemical quenching (a process that reduces ROS produced from excess excitation energy) in plants treated with paclobutrazol, indicating ROS levels were lower in plants treated with PGR. Abscisic acid (ABA) levels may also increase in

paclobutrazol-treated plants, stimulating stomatal closure and reducing water lost from evapotranspiration (Desta & Amare, 2021). Paclobutrazol serves as a stress protectant by enhancing the activity of enzymes and hormone levels while maintaining cell stability. The ability of paclobutrazol to provide drought tolerance could help expand the distribution of plants to consumers in more arid climates.

The PGR application method, concentration, and plant species affect how a plant responds to PGRs. PGRs are often applied as foliar sprays or substrate drenches. Bañón et al. (2002) compared the reduction in plant growth between *Dianthus caryophyllus* sprayed and drenched with a range of paclobutrazol concentrations. The study determined that drenches of lower concentration decreased plant height more than higher concentrations of foliar sprays; therefore, drenches were more efficient for *Dianthus caryophyllus* plants (Bañón et al., 2002). One reason for the efficiency of drenches could be that paclobutrazol is transported through the xylem, immobile in the phloem, and has low solubility in water (Desta & Amare, 2021; Ribeiro et al., 2011). Unlike drench applications, foliar spray applications do not take advantage of the movement of paclobutrazol through the xylem. Therefore, foliar sprays limit the interaction of paclobutrazol beyond the nearest tissue, producing more localized effects. Quinlan and Richardson (1986) reported that ^{14}C-paclobutrazol movement in apples was more prevalent in younger tissue (closer to shoot tips) than older tissue following spray applications, demonstrating the limited translocation of paclobutrazol after foliar sprays. The paclobutrazol also moved toward shoot tips rather than roots, consistent with the theory that the PGR is translocated through the xylem (Quinlan & Richardson, 1986). Additionally, the effect of paclobutrazol sprays dissipated quickly (Quinlan & Richardson, 1986), indicating application would need to be repeated throughout the season. Drench applications allow for an even distribution of

paclobutrazol throughout the plant, creating more uniform results. Applying paclobutrazol with drenches may also reduce labor costs due to the efficiency of singular applications compared to foliar sprays.

The concentration of PGR and plant species also affects the results of treatment. If the PGR concentration is too high, the treatment can produce plants with less consumer appeal. Al-Khassawneh et al. (2006) explored the effects of various concentrations of paclobutrazol on *Iris nigricans*. The study found that paclobutrazol concentrations higher than the optimal concentration reduced height to the point that plants were undesirable (Al-Khassawneh et al., 2006). Plants treated with concentrations above the optimal treatments also experienced a reduction in leaf number and delayed flowering (Al-Khassawneh et al., 2006). Dasoju et al. (1998) found a reduction in the inflorescence diameter of *Helianthus annuus* plants after treatment with a concentration higher than the optimum, which resulted in a less appealing product. Therefore, the concentration of PGR applied to plants influences the intensity of the plant response.

Additionally, species or variety can affect how plants react to PGRs, even when applied at the same concentration. Cavins et al. (2002) compared two *Solenostemon scutellarioides* cultivars after spraying plants at two different time points with uniconazole (another triazole-based PGR). The study determined that *Solenostemon scutellarioides* 'Burgundy Sun' required two sprays of uniconazole to control height. Alternatively, the height of *Solenostemon scutellarioides* 'Solar Storm' was efficiently reduced with one spray of the same concentration and severely reduced by the second spray (Cavins et al., 2002). Treatments must improve the growth habits of plants without reducing factors related to the marketability of the plants. For example, consumers prefer compact plants produced from PGR application; however, the flower

presence must also be maintained to ensure plants are appealing. Applying PGR concentrations higher than the optimal concentration for a species may reduce the plant's marketability. Concentrations higher than the optimum are also economically inefficient because more of the PGR is used, and fewer plants are likely to be sold. Determining the rates that significantly improve growth habit will ensure resources are used efficiently. *Pycnanthemum* is a relatively unexplored genus; therefore, identifying the optimal PGR concentration for *Pycnanthemum* will make the genus more accessible to growers and appealing to consumers.

Pycnanthemum virginianum and *Pycnanthemum flexuosum* are herbaceous perennials in the *Lamiaceae*. The genus *Pycnanthemum* is native to North America (Hummer et al., 2020) and is characterized by a mint or thyme-like fragrance. The inflorescences are arranged in a cyme composed of small tubular white or purple flowers speckled with purple dots. *Pycnanthemum virginianum* is 0.6 – 1.1 m and has 0.8 – 1.5 cm wide flowers (Chambers, 1961b). The species grows north of Georgia and out to North Dakota (Weakley, 2020). *Pycnanthemum flexuosum* grows between 0.5 – 1.2 m tall, with flowers that are 2 – 4 cm wide (Chambers, 1961b), and is endemic to the southeastern United States, stretching up to Virginia and out to Mississippi (Weakley, 2020). *Pycnanthemum* attracts pollinators and has the potential to appeal to the public. Developing procedures for *Pycnanthemum*'s production will make the genus more accessible for both growers and breeding programs.

Materials and Methods

Plant Material

Pycnanthemum virginianum and *P. flexuosum* from the USDA-ARS Western Regional Plant Introduction Station (Pullman, WA) were grown from seed and transplanted into 6.65 cm (280 mL) Deep Press Fit Pots (The HC Companies, Twinsburg, GA) filled with Pro-line C/B

Growing Mix (Shady Dale, GA) and used as stock plants for the cuttings taken to propagate the plants in the study. Once the propagules were established, the plants were transplanted into C300 2.8 L pots (Nursery Supplies Inc., Kissimmee, FL) with an outdoor mix composed of 20% peat moss, 28% 0.95 cm aged pine bark, 42% 1.59 cm aged pine bark, and 10% sand (Old Castle, Shady Dale, GA), and spaced 30 cm apart on a container pad. The experiment in 2022 received 16 g of Osmocote Plus 15-9-12, 8-9-month (15-4-10 N-P-K) (ICL Specialty Fertilizers, Summerville, SC) once they were placed on the container pad, while the experiment in 2023 was fertilized with 24 g of Osmocote Plus 15-9-12, 8-9-month (15-4-10 N-P-K) and watered twice daily by a sprinkler system. Once plants were established on the container pad, each plant was cut down to 4 cm before treatments were applied. Plants were cut 48 hours before treatment to give plants a chance to acclimate and were treated on June 9, 2023.

Treatments in 2022

A preliminary study compared *P. flexuosum* plants treated with 0, 0.25, 0.5, 1, and 2 mg a.i. of Bonzi®/pot (Greensboro, North Carolina), a commonly used plant growth regulator. Nine replications of each treatment were arranged in rows of increasing concentration. On the last day of collection (September 23, 2023), plants treated with 1 and 2 mg a.i. of Bonzi®/pot were smaller than the controls; however, 1 and 2 mg a.i./pot treatments were not significantly different from treatments with lower concentrations. Initial results informed additional treatments of *P. flexuosum* plants in 2023.

Treatments in 2023

The experiment in 2023 examined *P. virginianum* and *P. flexuosum* plants. The study in 2022 informed treatments of *P. flexuosum*, while *P. virginianum* was treated with a broad range of concentrations due to a lack of preliminary information. Plants were separated by species and

arranged in randomized complete blocks with three replicates per block. Plants were drenched with 118 ml of a Bonzi® and water mixture. Three blocks (nine samples per treatment) of *P. virginianum* were treated with 0, 0.5, 1, 2, 4, 8, and 16 mg a.i. of Bonzi®/pot. Two blocks (six samples per treatment) of *P. flexuosum* were treated with 0, 6, and 8 mg a.i./pot.

Data Collection

Biweekly measurements were recorded between June 2023 and August 2023 to determine if treatments affected plant growth, including width 1, width 2 (perpendicular to width 1), height, and average internode length. The height was measured from the soil surface to the highest branch. The width of plants was calculated by averaging width 1 and width 2. The volume was calculated by dividing the elliptical volume by 2 to represent a hemisphere ($V = (½) [(4/3) \pi (\text{height} * (1/2 \text{ width } 1) * (1/2 \text{ width } 2))]$). The growth index was calculated by averaging height, width 1, and width 2. The average internode length was calculated by measuring the length between the third and fourth internode on three branches to provide an average internode length per plant. The flowers were counted, and the vegetative growth was cut on the last day of data collection (August 21, 2023) to quantify flowering and dry weight. Plants were cut flush with the upper rim of the pots, placed in bags, and allowed to dry for three weeks in an empty greenhouse set to 25°C (daytime temperatures often exceeded 40°C) before being weighed. The density of plants was determined by dividing the shoot dry weight by the volume of plants on the final day of data collection.

Statistical Analysis

A repeated measures ANOVA (RStudio, PBC, Version 1.4.1725, Boston, Massachusetts) was used to analyze the data for significant treatment effects over the collection period. An ANOVA analysis (RStudio) was used to determine if the concentration of paclobutrazol affected

vegetative traits taken on the last day of data collection. The model for the height, volume, and dry weight of the *P. virginianum* plants was log-transformed to improve the model fit and ensure models met normality and homoscedasticity assumptions (Hopper et al., 1994; Sanz-Pérez & Castro-Díez, 2010). The height and growth index of the *P. flexuosum* plants were squared, while the density of the *P. flexuosum* plants was log-transformed to improve model fit and meet normality and homoscedasticity assumptions (Hopper et al., 1994; Sanz-Pérez & Castro-Díez, 2010). A Poisson regression (a generalized linear model used for discrete and positive response variables, such as count data) was used to compare flowering between *P. flexuosum* treatments (O'Hara & Kotze, 2010). *Pycnanthemum virginianum* data was analyzed using a non-binary regression due to a violation of the equal dispersion assumption. Outliers were identified using RStudio, characterized by values less than or greater than 1.5 times the standard deviation, and then winsorized. Winsorization replaces outliers with the value closest to the outlier, which normalizes the data without creating unequal sample sizes between treatments (Kwak & Kim, 2017). A Tukey HSD post-hoc test (RStudio) was used to compare the effects of treatments on *P. virginianum*. However, a Bonferroni correction (RStudio) was more fitting for *P. flexuosum* due to the comparison of fewer treatments during the experiment. Significance was reported as $p \leq 0.05$.

Results

The concentration of paclobutrazol significantly affected on the height and growth index of *P. virginianum and P. flexuosum* plants throughout the study. The growth of plants over time and the polynomial equations are displayed in Figure 3.1-3.4.

P. virginianum was shorter than the control plants in all treatments besides 0.5 mg a.i./pot. Paclobutrazol treatments greater than 0.5 mg a.i./pot reduced height by 33.7 – 47.6%

compared to the control (Table 3.1; also see Figure 3.5). *Pycnanthemum virginianum* drenched with 0.5, 1, 2, and 4 mg a.i./pot had more flowers than plants treated with 0, 8, or 16 mg a.i./pot. The flower abundance of plants treated with 0.5, 1, 2, and 4 mg a.i./pot increased by 84.0 – 141.2% compared to controls (Table 3.1), while higher concentrations were not different from the control. The shoot dry weight of *P. virginianum* drenched with 16 mg a.i./pot were 34.7% lower than the control (Table 3.1). Other treatments did not produce different shoot dry weights than the control (Table 3.1). No difference in growth index was present between treatments and the control. However, the growth index of plants treated with 0.5, 1, 2, and 4 mg a.i./pot were 6.6 – 10.1% larger than the control, while the 8 and 16 mg a.i./pot treatments had 14 and 11% smaller growth indexes compared to the control, respectively (Table 3.1). The density of plants treated with 8 mg a.i./pot increased plant density by 43.4%, although the density of plants treated with 16 mg a.i./pot were not different than the control (Table 3.1).

The heights of *P. flexuosum* plants treated with 6 and 8 mg a.i./pot were 41.9 and 43% lower than the control, respectively (Table 3.1; also see Figure 3.6). Plant height did not differ between plants drenched with 6 and 8 mg a.i./pot (Table 3.1). The control *P. flexuosum* had more flowers than the plants treated with 6 and 8 mg a.i./pot, which had 89.4 and 100% fewer flowers, respectively (Table 3.1). *Pycnanthemum flexuosum* controls had a higher shoot dry weight than plants treated with 6 mg a.i./pot, which were 52.2% lighter than the control (Table 3.1). The dry weight of plants treated with 8 mg a.i./pot did not differ from the control or plants treated with 6 mg a.i./pot (Table 3.1). Drenching *P. flexuosum* plants with 6 and 8 mg a.i./pot decreased the growth index by 37.1 and 31.8% compared to the control (Table 3.1). The plants treated with 6 and 8 mg a.i./pot also increased the vegetation density by 127.4 and 96.3% (Table 3.1).

Discussion

Paclobutrazol is a triazole that affects plant hormone levels. The triazole is commonly known for inhibiting gibberellin biosynthesis, the plant hormone responsible for stem elongation (Kurniawati et al., 2023). Stem elongation contributes to plant height; therefore, paclobutrazol limits plant size by reducing gibberellin synthesis. *Pycnanthemum flexuosum* and *P. virginianum* exhibited a decrease in plant height when comparing treated plants to control plants. Other members of the *Lamiaceae*, including *Mentha piperita* and *Ocimum basilicum* (Firdausa & Kurniawati, 2022; Santos Filho et al., 2022), have exhibited a similar reduction in plant height. The effect of paclobutrazol on the internodes of plants could be responsible for the decrease in height. Inhibiting gibberellin biosynthesis allows paclobutrazol to influence internode formation. Paclobutrazol may reduce the length or number of internodes (Hamid & Williams, 1997), which reduces plant height. Kasim et al. (2018) noted that the internode length of *Salvia splendens* decreased as the concentration of paclobutrazol increased. Kurniawati et al. (2023) demonstrated the role of internode count on plant height in *O. basilicum* when describing the reduction in *O. basilicum* height and the number of internodes as paclobutrazol concentrations increase. The internode length of *P. flexuosum* plants and most *P. virginianum* did not vary from controls despite differences in plant height (Table 3.2). The observed decrease in height without a similar trend in internode lengths suggests the number of internodes decreases rather than the length. Therefore, a decline in internode count could contribute to the reduced height of *P. flexuosum* and *P. virginianum*.

The PGR increased the flowering of *P. virginianum* at low concentrations and decreased the flowering at high concentrations (Table 3.1). However, all concentrations of paclobutrazol decreased the flowering of *P. flexuosum* plants (Table 3.1). Previous studies have observed an

increase and decrease in flowering following paclobutrazol treatment. For example, ornamental plants such as *Osteospermum ecklonis* experienced more flowering after paclobutrazol treatment (Olsen & Andersen, 1995), while flower abundance in *Dianthus caryophyllus* and *Ocimum sanctum* decreased (Bañón et al., 2002; Divya Nair et al., 2009). The observed increase in flowering could be due to the ability of paclobutrazol to increase chlorophyll contents. Applications of paclobutrazol increased chlorophyll content in *Salvia splendens* and *Capsicum chinense* (França et al., 2017; Kasim et al., 2018), which could improve light capture and potential plant growth. On the other hand, the flowering of *P. virginianum* and *P. flexuosum* plants decreased at the highest concentrations (8 and 16 mg a.i./pot and 6 and 8 mg a.i./pot, respectively) (Table 3.1). High concentrations of paclobutrazol may reduce flower abundance by inhibiting gibberellin synthesis. *Prunella vulgaris* treated with gibberellin 3 exhibited an increase in flowering (Li et al., 2022). Similarly, *Matricaria chamomilla* flowering increased due to applications of gibberellic acid. Gibberellin plays a role in promoting flowering; therefore, inhibiting gibberellin could explain the reduction of flowering. The inhibition of gibberellin does not occur at the same magnitude when applying low concentrations of paclobutrazol, which could explain the increase in flowering compared to higher concentrations.

The shoot dry weight of *P. virginianum* decreased as paclobutrazol concentrations increased. *Pycnanthemum flexuosum* plants exhibited a similar decrease at 6 mg a.i./pot, although the dry weight increased slightly at 8 mg a.i./pot. Paclobutrazol treatments could reduce dry weight by restricting the number of leaves or leaf area. Singh et al. (2008) described a decrease in *Salvia sclarea* leaf number and suggested the reduction in leaf number resulted in a decrease in the shoot dry weight. Divya Nair et al. (2009) reported a smaller leaf area in *Ocimum sanctum* following paclobutrazol drenches and abscisic acid (ABA) treatment. The similar

decrease of *O. sanctum* leaf area in ABA treatments compared to paclobutrazol treatments indicates an increase in ABA concentration due to paclobutrazol may result in smaller leaves, reducing the shoot dry weight of plants. Similarly, increasing ABA concentrations in *Dracocephalum moldavica* decreased leaf area under well-watered and drought conditions (Khaleghnezhad et al., 2021). Paclobutrazol inhibits gibberellin, which responds antagonistically to ABA; therefore, reducing gibberellin synthesis could allow ABA concentrations to increase, restricting leaf production. The slight increase in the dry weight of *P. flexuosum* plants treated with 8 mg a.i./pot could be due to the complete inhibition of flowering, allowing more resources to be directed to vegetative growth rather than reproductive growth.

The density of *P. virginianum* plants was highest for plants drenched with 8 mg a.i./pot (Table 3.1). The volume of plants treated with 8 mg a.i./pot was smaller than the control plant, while shoot dry weight did not differ, resulting in greater density (Table 3.2). Paclobutrazol limits the synthesis of gibberellin, which reduces the elongation of cells and decreases plant volume. The volume of *P. virginianum* plants treated with 16 mg a.i./pot was not different from 8 mg a.i./pot treatments or controls; however, the shoot dry weight of plants treated with 16 mg a.i./pot was lower than control plants (Table 3.1). Therefore, the plants drenched in 16 mg a.i./pot had a lower density than those treated with 8 mg a.i./pot due to a lighter dry weight (Table 3.1). The difference in shoot dry weight could be due to smaller or fewer leaves on plants treated with 16 mg a.i./pot, caused by paclobutrazol inhibiting vegetative growth. Paclobutrazol reduced leaf area and number following treatments of *Salvia officinalis*, which reduced overall shoot dry weight (Bañón et al., 2023). Additionally after paclobutrazol treatments, *P. virginianum* may redirect resources from aboveground into belowground tissue (Jaleel et al.,

2008), which reduces the shoot dry weight. An increase in belowground growth redirects plant resources to roots, limiting shoot growth and reducing the shoot dry weight and density of plants.

Despite a decrease in shoot dry weight, the density of treated *P. flexuosum* increased (Table 3.1). Teto et al. (2016) found a reduction in shoot dry weight and plant volume in paclobutrazol-treated *Leonotis leonurus*, resulting in a higher vegetative density after treatment. Plant volume declined after paclobutrazol application due to the inhibition of cell elongation. Increasing plant density requires a lower volume than shoot dry weight; therefore, plant volume must decrease by a higher degree than shoot dry weight to produce compact plants. The volume of treated *P. flexuosum* was also smaller than control plants (Table 3.1). Therefore, the reduction in plant volume contributed to the increased density of *P. flexuosum*.

The growth index of *P. virginianum* increased and then decreased, as evident by the decrease when comparing low concentrations to high concentrations (Table 3.1). The initial increase in growth index at lower concentrations may indicate drought stress. Paclobutrazol helps plants tolerate environmental stress, including drought (Berova et al., 2002). Drought tolerance promotes more growth in treated plants under water deficit than controls. Paclobutrazol protects plants from drought stress by promoting antioxidant enzyme activity, enhancing osmotic adjustment by accumulating soluble sugars, and reducing water loss (Bañón et al., 2023; Fan et al., 2020). Limiting damage from environmental stress improves plant growth compared to controls. Paclobutrazol applications also increase chlorophyll content (Gopi et al., 2007), which allows plants to accumulate more resources for plant growth. The ability of paclobutrazol to protect plants from stress while enhancing growth results in larger plants than the control; however, paclobutrazol also limits cell elongation. At high concentrations, the PGR limits

growth despite environmental conditions, explaining the decrease in the growth index when *P. virginianum* and *P. flexuosum* were treated with higher concentrations of the PGR.

Paclobutrazol is a commonly used PGR for ornamental plants, capable of producing compact and fuller-looking plants, which appeals to consumers and assists growers. Determining the optimal concentrations of paclobutrazol will improve the accessibility of *Pycnanthemum*. *Pycnanthemum flexuosum* and *P. virginianum* species exhibited the desired decrease in height; however, both also experienced a decrease in flowering at higher concentrations. Concentrations below 6 mg a.i./pot should be examined for *P. flexuosum,* while *P. virginianum* may benefit from paclobutrazol concentrations between 4 and 8 mg a.i./pot to find a balance between increasing flowering and improving density. Examining a range of PGR concentrations informs growers' strategies, ensuring plants are desirable to consumers and efficient for production. Concentrations too low produce leggy plants, prone to lodging, while applying high concentrations can reduce flowering and waste resources. Paclobutrazol also protects plants against drought by decreasing transpiration, limiting the concentration of reactive oxygen species, and encouraging root elongation. Growers continuously face environmental stressors in ornamental production; therefore, providing additional tools to combat stress is valuable to growing programs. Exploring the effects of paclobutrazol at different concentrations allows growers to optimize PGR applications for consumer appeal.

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Tables and Figures

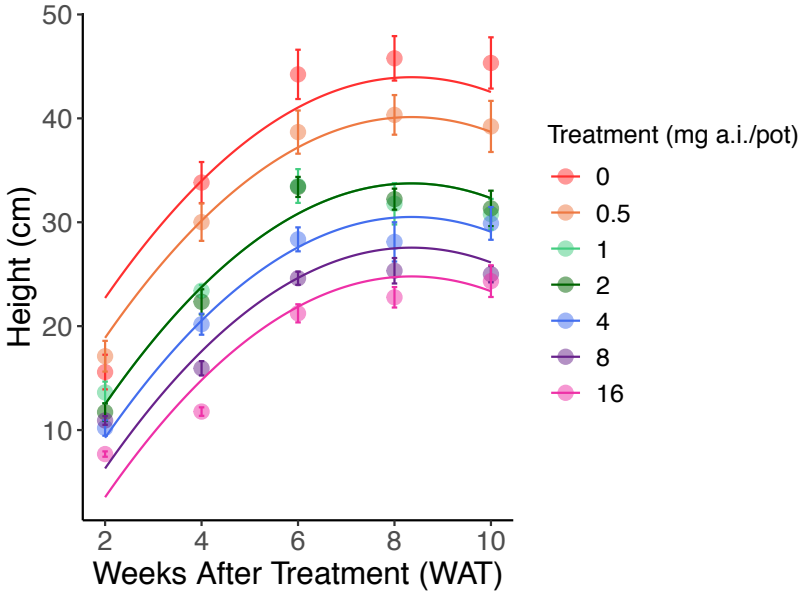

Figure 3.1. The height of *P. virginianum* after paclobutrazol treatments. The quadratic equation can be used to predict the effects of additional paclobutrazol concentrations: 0 mg a.i./pot ($y = -3.3025x^2 + 26.961x - 7.6053$), 0.5 mg a.i./pot ($y = -2.503x^2 + 20.473x - 0.8169$), 1 mg a.i./pot ($y = -2.3833x^2 + 18.568x - 2.8711$), 2 mg a.i./pot ($y = -2.4786x^2 + 19.726x - 5.82$), 4 mg a.i./pot ($y = -1.7738x^2 + 15.373x - 3.2578$), 8 mg a.i./pot ($y = -1.1787x^2 + 10.896x + 0.3764$), and 16 mg a.i./pot ($y = -0.9952x^2 + 10.44x - 2.7956$).

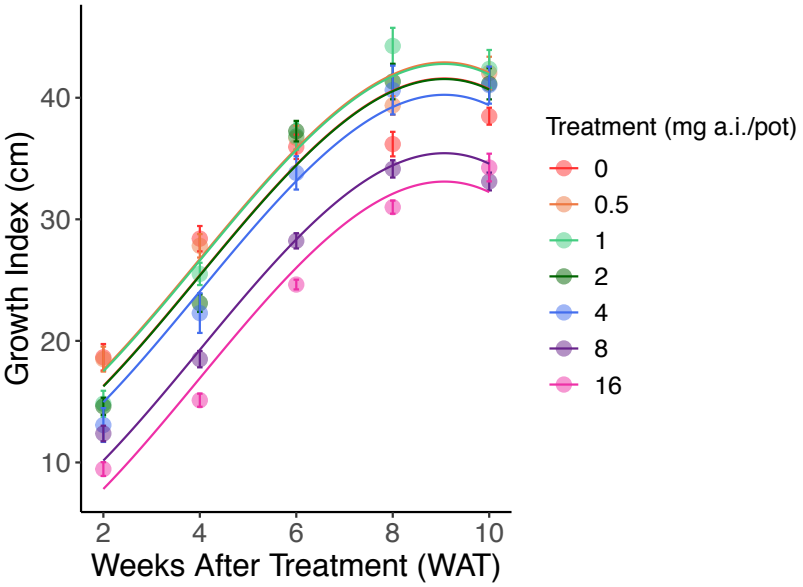

Figure 3.2. The growth index of *P. virginianum* after paclobutrazol treatments. The cubic equation can be used to predict the effects of additional paclobutrazol concentrations: 0 mg a.i./pot ($y = 0.2727x^3 - 4.0083x^2 + 20.552x + 1.6006$), 0.5 mg a.i./pot ($y = 0.036x^3 - 1.7181x^2 + 15.077x + 4.9351$), 1 mg a.i./pot ($y = -0.7321x^3 + 4.4746x^2 + 2.7326x + 8.4015$), 2 mg a.i./pot ($y = -0.8275x^3 + 5.4896x^2 - 0.6534x + 10.302$), 4 mg a.i./pot ($y = -0.7275x^3 + 4.8634x^2 + 0.3612x + 8.4504$), 8 mg a.i./pot ($y = -0.8402x^3 + 6.5638x^2 - 8.0738x + 14.725$), and 16 mg a.i./pot ($y = -0.5799x^3 + 4.6512x^2 - 3.7281x + 9.0119$).

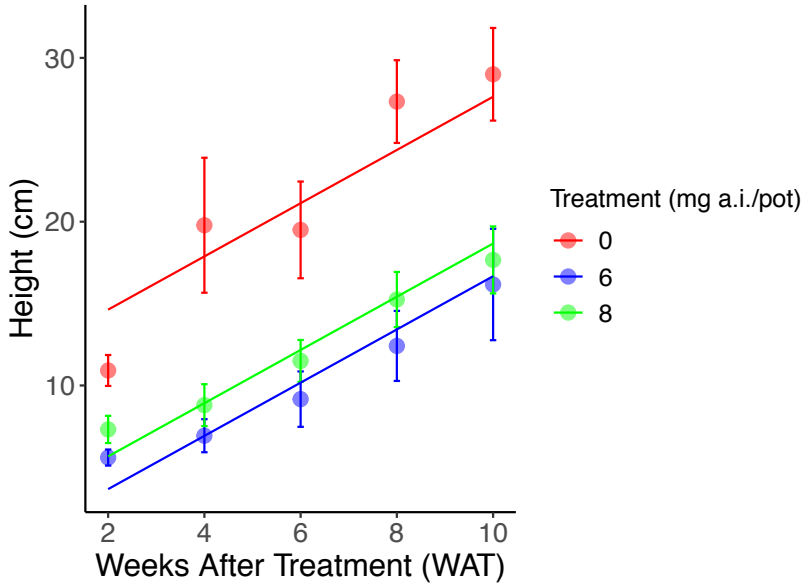

Figure 3.3. The height of *P. flexuosum* after paclobutrazol treatments. The linear equation can be used to predict the effects of additional paclobutrazol concentrations: 0 mg a.i./pot (y = 4.7717x + 7.3917), 6 mg a.i./pot (y = 2.76x + 2.2033), and 8 mg a.i./pot (y = 2.715x + 3.9617).

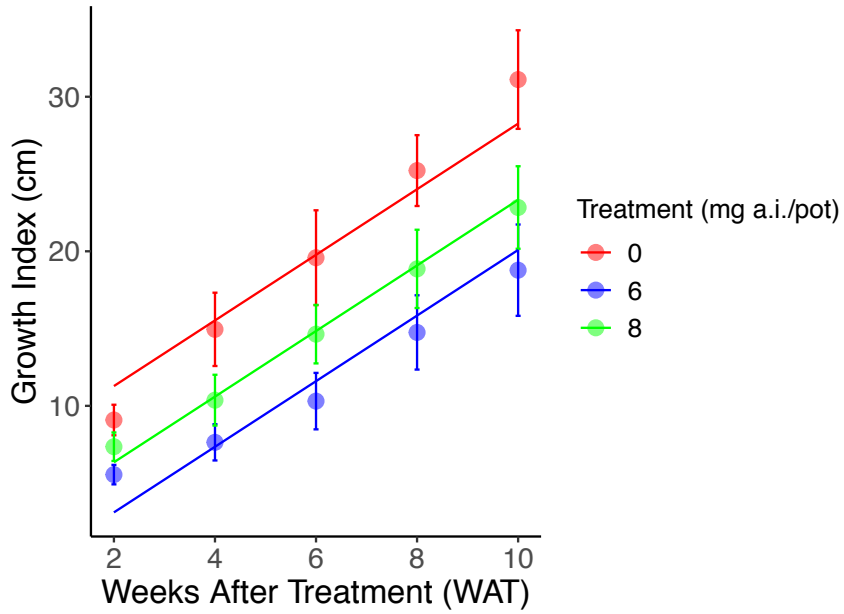

Figure 3.4. The growth index of *P. flexuosum* after paclobutrazol treatments. The linear equation can be used to predict the effects of additional paclobutrazol concentrations: 0 mg a.i./pot (y = 5.5661x + 3.425), 6 mg a.i./pot (y = 3.5339x + 1.2339), and 8 mg a.i./pot (y = 3.945x + 2.9761).

Figure 3.5. *Pycnanthemum virginianum* 10 weeks after paclobutrazol drench. Plants were treated with 0, 0.5, 1, 2, 4, 8, and 16 mg a.i./pot of Bonzi® (left to right).

Figure 3.6. *Pycnanthemum flexuosum* 10 weeks after paclobutrazol drench. Plants were treated with 0, 6, and 8 mg a.i./pot of Bonzi® (left to right).

Table 3.1. The average of *Pycnanthemum* growth and reproductive characteristics following paclobutrazol treatments. Data shown is from the last day of data collection (August 21, 2023).

	Treatment (mg a.i./pot)	Height (cm)	Growth Index (cm)	Flowering	Dry Weight (g)	Density (mg/cm3)
Genus *Pycnanthemum*						
P. virginianum						
	0	46.4 a	38.5 ab	21.6 a	32.7 ab	1.2 a
	0.5	39.2 ab	42.0 a	39.7 b	37.0 a	1.0 a
	1	30.8 bc	42.4 a	45.1 bc	39.3 a	1.1 ab
	2	31.3 bc	41.1 a	49.8 c	35.2 ab	1.0 a
	4	29.1 bcd	41.0 a	52.0 c	34.7 ab	1.1 a
	8	25.0 cd	33.1 b	26.4 a	28.4 b	1.7 b
	16	24.3 d	34.3 b	26.7 a	21.3 c	1.4 ab
P. flexuosum						
	0	31.0 a	33.5 a	119.8 a	26.5 a	1.3 a
	6	18.0 b	21.1 b	12.7b	12.7 b	3.0 b
	8	17.7 b	22.8 b	0.0b	16.3 ab	2.6 b

Table 3.2. The average of *Pycnanthemum* internode lengths and volumes following paclobutrazol treatments. Data shown is from the last day of data collection (August 21, 2023).

	PBZ (mg a.i./pot)	Internode length	Volume (m3)
Genus *Pycnanthemum*			
P. virginianum			
	0	0.9 a	287.3 ab
	0.5	1.1 ab	386.4 a
	1	1.0 a	378.0 a
	2	1.4 b	344.8 a
	4	1.3 ab	347.4 a
	8	1.4 ab	180.9 c
	16	1.2 ab	199.8 bc
P. flexuosum			
	0	2.33 a	175.4 a
	6	1.91 a	44.1 b
	8	2.22 a	71.2 b

CHAPTER 4

DETERMINING THE GENOME SIZE OF *PYCNANTHEMUM* SPECIES

[1]Swiantek K. and J. Ruter. To be submitted to HortScience

Abstract

Breeding for pollinator support and consumer appeal fosters public interest in pollinator protection. *Pycnanthemum* is a native pollinator plant with market potential that could be introduced into landscapes to reconnect fragmented pollinator habitats. Cultivation of *Pycnanthemum*, however, is difficult due to unclear phylogeny and current identification methods. Little genetic information exists for the genus; therefore, the current understanding of phylogenic grouping is based on a combination of chromosome number, proposed ploidy level, compatibility, and morphology. Morphological differences between *Pycnanthemum* species can be difficult to distinguish; therefore, genome size could be used to improve identification accuracy. Flow cytometry was used to determine various *Pycnanthemum* species' genome sizes and base pair compositions. The DNA content of *Pycnanthemum* was determined using 4′,6-diamidino-2-phenylindole (DAPI), while the base pair composition was determined by comparing DAPI and propidium iodide (PI) to ensure DAPI could accurately estimate the genome size. The study found the DNA content (2C value) of *Pycnanthemum* ranged from 2.89 ± 0.08 pg (*P. californicum*) to 7.22 ± 0.24 pg (*P. torreyi*). A linear regression indicated that genome size could be predicted according to the ploidy levels and chromosome numbers proposed by previous research. The GC content between the *Pycnanthemum* species tested ranged from 29.32% (*P. incanum*) to 34.47% (*P. setosum*) and did not differ from standards, indicating the genome size estimations with DAPI properly reflect DNA content. Genome size information could be used to identify species, clarify ploidy levels, and infer compatibility. Breeding programs would also benefit from genetic knowledge, which could inform hybridization decisions.

Introduction

Pycnanthemum, also known as mountain mint, is an herbaceous perennial member of the *Lamiaceae*, native to North America. *Pycnanthemum albescens, P. beadlei, P. curvipes, P. flexuosum, P. floridanum, P. incanum, P. loomisii, P. muticum, P. nudum, P. pilosum, P. pycnanthemoides, P. setosum, P. tenuifolium, P. torreyi, P. verticillatum*, and *P. virginianum* are native to the central and eastern region of the U.S., while *P. californicum* originates from California (Hummer et al., 2020). Most *Pycnanthemum* plants have an expansive native and current range spanning multiple states (Kartesz, 2015). However, *P. setosum* is considered rare in several states, while certain states consider *P. torreyi* and *P. pycnanthemoides* endangered (Williams, 2005).

The genus is a pollinator-attractive plant (Bailey, 2022; Cadotte et al., 2017; MacLeod et al., 2020; Porter, 2010) with potential consumer appeal that could help improve the accessibility of pollinator conservation efforts to the public. *Pycnanthemum* does not depend on specific pollinators, suggesting the genus is a generalist, open to attracting a variety of pollinators (Williams, 2005). MacLeod et al. (2020) found that rare and crop-pollinating bees visited *P. tenuifolium* most, compared to other pollinator-attractive genera. Similarly, beneficial insects were collected on *P. flexuosum* more often than other pollinator plants (Porter, 2010). The olfactory signals compounds and quantity of nectar and pollen of *Pycnanthemum* may improve resource accessibility and potentially influence the abundance of visitors.

Consumers would appreciate the fragrance of *Pycnanthemum*, which emits a mint or thyme-like aroma. The purple-speckling on the white or light purple flowers is also striking and draws attention to the inflorescence. *Pycnanthemum* species are native to various environments, including moist to dry soils and rocky or forest environments (Hill, 2007). The adaptability of

the genus to different growing conditions also makes the plant an appealing candidate for cultivation. The genus has adapted to a various environmental conditions, which allows the plant to be distributed to a wider market. Additionally, *Pycnanthemum*'s status as a native pollinator plant attracts consumers interested in native plants and pollinator conservation. Consumers who purchase native plants prefer plants with fewer care requirements and inputs (Campbell et al., 2017). Therefore, *Pycnanthemum's* phenotypic appeal and ability to tolerate diverse conditions increase the size of the target market and garner consumer interest.

Cultivation, however, can be difficult due to confusion involving identification and phylogeny. Determining the genome size of the genus would inform hybridization decisions, streamline cultivation, and improve identification. Interspecific hybridizations of *Sarcococca* were most successful (more than 50% of hybrids producing fruit) between plants with similar genome sizes and compatible ploidy levels (Denaeghel et al., 2017). Therefore, breeders can make efficient hybridization decisions when genome sizes are known. Chambers and Chambers (1971) found that plants with different ploidy levels successfully produced hybrids, though most hybrids developed from tetraploid and diploid crosses were sterile triploids. Unlike ploidy, chromosome number was labeled a barrier to hybridization between some *Pycnanthemum* species by Chambers and Chambers (1971). Chromosome number can be used to devise hybridization strategies in species such as *Callicarpa* (Contreras & Ruter, 2011), although chromosome number is unrelated to the success of crosses in other genera, including *Salvia* (Tychonievich & Warner, 2011). Therefore, the genome size can provide a better understanding of compatibility between species.

Due to a lack of genetic information, previous studies have attempted to group *Pycnanthemum* species into related-species groups, which would potentially cross more readily

(Harlan & de Wet, 1971). Previously, Grant and Epling (1943) proposed groups based on morphological traits – sorting *Pycnanthemum* into the Incanum Phylad, the Virginianum Phylad, and periphery groups distantly linked to the two main groups. However, Chambers (1993) found that many plants from different groups hybridized successfully and suggested the proposed classifications did not predict compatibility. Weakley (2015) highlights the disorder of *Pycnanthemum's* evolution when claiming that the genus evolved into distinct species prior to the development of polyploidy, suggesting some species exist in different forms. Additionally, the maturity of plants can also influence the ease of identification. Wofford (1989) and Snyder (1994) discuss using calyxes to identify *Pycnanthemum* species. However, calyxes only develop once plants transition into reproductive growth, and many *Pycnanthemum* species remain mainly vegetative during the first year of growth (Dr. Svoboda V. Pennisi, personal communication). Therefore, morphology-based classifications cannot efficiently predict compatibility between *Pycnanthemum* species.

Chambers and Chambers (2008) altered the proposed grouping into seven sections (*Pycnanthemum*, *Aristatae*, *Brachystemum*, *Capitellatae*, *Macrocephalae*, *Nudae*, *Californicae*) based on morphology, ploidy level, chromosome number, and hybridization success. Chambers (1961b) and Chambers and Chambers (1971) reported the chromosome numbers of *Pycnanthemum* species, suggesting the genus has a mixture of basic chromosome numbers (x = 18, 19, 20,) and ploidy levels (2n = 2x, 4x, 5x). Chambers and Hamer (1992) also proposed a hexaploid *P. torreyi* with x = 20. Mitotic and meiotic cells were studied from root and pollen mother cell squashes to determine the chromosome number and estimate the ploidy levels of *Pycnanthemum* species (Chambers, 1961a; Chambers & Chambers, 1971). The variety of base chromosome numbers may indicate dysploidy or aneuploidy occurred during the evolution of

Pycnanthemum. Dysploidy is the gain or loss of chromosomes through chromosomal transformation, resulting in the separation or merging of chromosomes, while aneuploidy adds or removes single chromosomes mainly through errors in the separation of chromatids during cell division (Mayrose & Lysak, 2021). Similarly, the multiple base chromosome numbers present in *Callicarpa* and *Salvia* have been attributed to dysploidy and aneuploidy (Contreras & Ruter, 2011; Eroğlu et al., 2021). The possibility of dysploidy or aneuploidy further complicates phylogeny. Chambers and Chambers (2008) separated *Pycnanthemum* species into seven sections according to the compatibility of plants in a hybridization study conducted by Chambers (1993). The classifications grouped diploid species with morphologically and cytogenetically similar alloploids (Chambers, 1993).

Estimating the genome size of *Pycnanthemum* species could help verify the current understanding of *Pycnanthemum* classification, inform breeding programs, and improve the accuracy of identification. For example, Jedrzejczyk and Rewers (2018) confirmed most of the proposed *Mentha* classifications by calculating the genome size of *Mentha* species. Similarly, the proposed subgenera of *Geranium* were also verified using genome sizes (Akbarzadeh et al., 2021). Using genome size to determine whether the proposed groupings of *Pycnanthemum* are accurate will allow breeders to infer the genetic distance between groups, informing hybridization.

Potential ploidy levels can also be suggested by comparing the genome size and chromosome number, as seen in *Callicarpa* (Contreras & Ruter, 2011), which can verify the ploidy levels proposed by Chambers and Chambers (1971). Additionally, determining if a relationship exists between the ploidy level or chromosome number and the genome size allows breeders to predict the genome sizes of species and the success of interspecific hybridization.

However, the relationship between ploidy level or chromosome number and genome can differ between classifications, as seen in the subgenera of *Lavandula* (Van Oost et al., 2021). The relationship between ploidy level or chromosome number and genome size also depends on the genus. Tamura et al. (1998) found that genome size could be confidently predicted using the ploidy level of *Diospyros*. However, Choi et al. (2020) found no connection between the genome size of *Iris* and the chromosome number. Therefore, determining the relationship between genome size, chromosome number, and ploidy in *Pycnanthemum* would help determine if the current reported ploidy levels and chromosome numbers can predict genome size.

Flow cytometry is commonly used to determine the genome size of plants. The technique compares the fluorescence of a standard of known genome size to a sample after staining the base pairs of the plant material. The flow cytometer pulls stained cells through a small tube, where they are excited by a UV light or laser (Ochatt, 2006). As the samples run through the flow cytometer, the instrument graphs the fluorescence in a plot of fluorescence versus cell count (Ochatt, 2006). Then, the genome size can be calculated from the output of the flow cytometer (the mean fluorescence of the standard and sample peaks). Genome size is often reported as 2C, 1C, and 1Cx values. The 2C value is the amount of DNA in a somatic cell, the 1C value equates to the DNA in a nonreplicated gametic cell, while the 1Cx value is the amount of DNA in an unreplicated set of base chromosomes (Sliwinska et al., 2022). Calculating the 1Cx value allows for comparisons of genome size independent of ploidy.

Propidium iodide (PI) and 4′,6-diamidino-2-phenylindole (DAPI) are common fluorescent chemicals used for staining samples for flow cytometry. DAPI binds to the adenine and thymine base pairs, while PI intercalates with DNA strands and binds with all four base pairs (Contreras & Shearer, 2018). DAPI binds to fewer base pairs than PI, though previous studies

have shown that DAPI can provide accurate genome size estimates. Parris et al. (2010) determined the genome size of *Magnolia* species using DAPI and PI and found no significant difference between the reported values. However, a separate study determined that the DNA estimates of a various plants treated with DAPI and PI significantly differed (Doležel et al., 1992). Ortega-Ortega et al. (2019) suggest that the binding properties of PI and DAPI and the unequal base composition between standards and samples are responsible for observed differences in genome size estimation. Ultimately, the reliability of DAPI depends on the standard and sample having similar GC contents (Dolezel, 1997). DAPI preferentially binds to AT base pairs, while PI binds to all base pairs, meaning plants with similar AT contents will likely provide similar estimates with DAPI as PI. Therefore, the base pair composition of plants must be examined to determine whether DAPI is an appropriate tool.

Determining the genome size of *Pycnanthemum* would help develop breeding programs and cultivate a deeper understanding of the genus. Species with similar genome sizes are more likely to successfully hybridize. Additionally, verifying phylogenic relationships between *Pycnanthemum* species would indicate compatibility. The current groupings of *Pycnanthemum* are based on chromosome number, morphology, and compatibility. Using the genome size to distinguish between *Pycnanthemum* species would improve the accuracy and speed of identification. Reporting the genome sizes of *Pycnanthemum* encourages the cultivation of the genus by supplying information that predicts the success of hybrids and informs breeding programs.

Materials and Methods

Plant Material

Fourteen species of *Pycnanthemum* were collected and analyzed using flow cytometry. A single plant was used to represent each species to determine the interspecific variation in genome size among *Pycnanthemum* species. The germplasm used during the study are listed in Table 4.1.

Plants were maintained at the University of Georgia's Durham Horticulture Farm (33.944507, -83.375774) in a greenhouse set to keep temperatures at 25°C /20 °C and humidity at 40% / 30% (day/night conditions). Seed procured from nurseries and germplasm repositories were sown, then transplanted into 6.65 cm (280 mL) Deep Press Fit Pots (The HC Companies, Twinsburg, GA) in Pro-line C/B Growing Mix (Shady Dale, GA). Seedlings were transplanted into 6.65 cm (280 mL) pots with Pro-line C/B Growing Mix (Shady Dale, GA), left under 70% shade cloth with 21°C bottom heat, and misted every five minutes for five seconds. Plants collected from botanizing were propagated with cuttings treated with 3,000 mg/L Indole-3-butyric acid (IBA) solution and placed in 200 cell (14.6 mL) plug trays (Grower's Nursery Supplies, Salem, OR) in a 1:1 ratio of Pro-line C/B Growing Mix (Shady Dale, GA) to perlite. Cuttings were placed under 70% shade cloth with 21°C bottom heat and misted every five minutes for five seconds. Plants were left under shade and mist until roots established (approximately eight weeks). All collected plant material was used as stock plants. Five cuttings were taken from each of the 14 stock plants and treated as previously stated. Once plants had established in 200 cell (14.6 mL) plug trays, they were transplanted to 11.43 cm (956 mL) Kordlock Square Pots (The HC Companies, Twinsburg, GA) and given 8 g of Osmocote Plus 15-9-12, 8-9-month (15-4-10 N-P-K) (ICL Specialty Fertilizers, Summerville, SC). In a greenhouse, plants were fertilized weekly with 200 mg/L of Jack's Professional Water Soluble

Fertilizer 20-10-20 Peat-Lite (20-4.4-16.6 N-P-K) (JR Peters Inc., Allentown, PA). These plants acted as the material for the following study.

Solanum lycopersicum 'Stupické' and *Pisum sativum 'Ctirad'* seeds were planted in Pro-line C/B Growing Mix (Shady Dale, GA) in 11.43 cm (956 mL) Kordlock Square Pots (The HC Companies, Twinsburg, GA). Plants were maintained in a greenhouse similar to the *Pycnanthemum* plants.

Determine the genome size of *Pycnanthemum*

Flow cytometry was used to estimate the genome size of each *Pycnanthemum* species. The peaks of the *Pycnanthemum* species were compared to a plant with a known genome size to calculate the genome. The genome size of *Pycnanthemum* differed between species, meaning certain species required a different standard. After preliminary testing, *Solanum lycopersicum* and *Pisum sativum* served as standards with 2C values of 1.96pg and 9.09 pg, respectively (Praça-Fontes et al., 2011). Standards were chosen based on the location of standard peaks to the sample peaks (Table 4.2). The genome sizes of the standard and sample needed to be similar enough that peaks could be viewed in the same window, though not so similar that peaks overlapped. For samples using *P. sativum* as a standard, 5 cm^2 of leaf tissue was removed from the *Pycnanthemum* and 5 cm^2 from *P. sativum*. Samples that required *S. lycopersicum* used 0.5 cm^2 of *S. lycopersicum* leaf tissue and 5 cm^2 of *Pycnanthemum* due to the greater concentration of DNA in *S. lycopersicum* samples compared to *Pycnanthemum* samples. Samples were taken from the newest leaf tissue and processed according to a Sysmex Cystain kit (Sysmex America, Inc., Lincolnshire, IL), using either PI or DAPI. The samples were analyzed using a CytoFlex flow cytometer (Beckman Coulter, Hialeah, FL) located in the Paul D. Coverdell Center for Biomedical and Health Science (University of Georgia Cytometry Shared Resource Laboratory,

Athens, GA) with a minimum of 1000 nuclei per sample. After running the samples through a flow cytometer, the data was uploaded into FlowJo (FlowJo 10.8.1, Ashland, OR); the genome size of the unknown (the *Pycnanthemum* species) was determined from the equation in Figure 4.1.

Staining with PI

A Sysmex Cystain PI Absolute P kit (Sysmex America, Inc., Lincolnshire, IL) was used when processing the samples stained with propidium iodide. The kit included a nuclei extraction buffer solution, RNase, Propidium iodide (PI), and a staining buffer. A staining solution was mixed using 12µl RNase, 24µl PI, and 250µl staining buffer (solution for 1 sample). The standard and *Pycnanthemum* leaf samples were chopped together with a razor into the nuclei extraction buffer solution. After chopping, the tissue and 500µl of the nuclei extraction buffer sat for 1 minute. Once the tissue sat in the solution, the large tissue was filtered from the solution by pipetting the mixture through a 50µm mesh filter (Sysmex America, Inc., Lincolnshire, IL) into a test tube. Then, 250µl of the staining solution was combined with the filtered mixture. The mixture was left at room temperature for 30 minutes and then placed in the fridge for another 90 minutes. Finally, the mixture was pipetted into a well on a 96-well plate and ran through the flow cytometer. The flow cytometer was set to display wavelengths between 488 – 690 nm. The processing of samples described above was used for each species of *Pycnanthemum*.

Staining with DAPI

A Sysmex Cystain UV Precise P kit (Sysmex America, Inc., Lincolnshire, IL) was used when staining samples with DAPI. The kit included a nuclei extraction buffer solution and a staining buffer. The standard and *Pycnanthemum* leaf samples were chopped together with a razor into a nuclei extraction buffer solution. After chopping, the rest of the 500µl nuclei

extraction buffer was added to the tissue. The mixture sat at room temperature for 5 minutes. Then, the mixture was pipetted through a 50µm mesh filter (Sysmex America, Inc., Lincolnshire, IL) into a test tube. The filtered mixture was stained using 250µl staining buffer, which contained DAPI. Finally, the sample was pipetted into a well on a 96-well plate and placed into the flow cytometer. The flow cytometer was set to display wavelengths between 405 – 450 nm. The processing of samples described above was used for each species of *Pycnanthemum*.

Comparing the Base Pair Composition

The equation in Figure 4.2. was used to determine whether the base pair composition was similar between the internal standard and the *Pycnanthemum* samples (Parris et al., 2010). The binding length of DAPI is estimated to be 3.5 bps (Parris et al., 2010), while the AT% of *Solanum lycopersicum* and *Pisum sativum* is 66% and 62.5%, respectively (National Center for Biotechnology Information, Bethesda, MD). *Pycnanthemum albescens*, *P. beadlei*, *P. floridanum*, *P. incanum*, P. *loomisii*, *P. muticum*, *P. pycnanthemoides*, and *P. setosum* were used to comapre the GC content of *Pycnanthemum* species to standards.

Statistical Analysis

Genome size was analyzed using analysis of variance and accepted if the coefficient of variance was less than 5% (Microsoft Excel, Version 16.54, Microsoft Corporation). A Bonferroni correction (RStudio, PBC, Version 1.4.1725, Boston, Massachusetts) was used to compare the genome sizes between *Pycnanthemum* species. The relationship between the experimental 2C values, and reported ploidy levels or published chromosome numbers (Chambers & Chambers, 1971) was analyzed using linear regression (Microsoft Excel, Version 16.54, Microsoft Corporation). Significance was reported as $p \leq 0.05$.

Results and Discussion

Genome size, Chromosome Number, and Ploidy Level

The estimated 2C genome size, chromosome number, proposed ploidy levels, and the 1Cx values of *Pycnanthemum* are listed in Table 4.2. According to Leitch et al. (1998), 61.5% of the *Pycnanthemum* species sampled were small (C value is less than 3.5 pg), while the other 38.5% had C values larger than 3.5 pg but smaller than 14 pg (the size of a large genome). The 2C genome size of each species did not differ from other species except for *P. albescens*, *P. nudum*, and *P. californicum*. *Pycnanthemum albescens* has a different 2C genome size than *P. virginianum, P. torreyi,* and *P. muticum*. Additionally, the 2C genome size of *P. californicum* differed from *P. flexuosum*, while *P. nudum* differed from *P. setosum*. The genome sizes within each section proposed by Chambers and Chambers (2008) were not different, according to the samples used in the study, implying the suggested categorization may indicate relatedness between species. However, the 1C values of *P. virginianum* and *P. torreyi* differed despite Chambers and Chambers (2008) grouping the species together. *Pycnanthemum virginianum* is a proposed tetraploid, while *P. torreyi* may be a tetraploid or hexaploid (Chambers & Hamer, 1992). According to the linear equation determined during the study (Figure 4.3), the *P. torreyi* in this study was a hexaploid, which could explain the significantly different 1C values and suggests hexaploid *P. torreyi* may be less compatible with *P. virginianum* as the tetraploid form. The similar genome sizes and compatibility between species outside of the proposed sections may also indicate relatedness between sections. *Pycnanthemum nudum* and *P. flexuosum* were also sorted into different sections due to the geographical distance restricting hybridization between the species; however, the species naturally hybridized (Chambers & Chambers, 1971), and the genome sizes were similar, which suggests the two species may be closely related.

The ploidy levels and chromosome numbers reported by Chambers and Chambers (2008) and 2C genome size (Figure 4.3 and 4.4) were strongly related (R^2 = 0.70 and 0.74, respectively). Similarly, Hamid and Williams (1997) reported a strong correlation between ploidy level and genome size of three geographically separate *Thymus* species. The relationship between the ploidy level and genome size was also indicted in the 1Cx values of *Pycnanthemum* species. The 1Cx values compare the monoploid genome of plants (Sliwinska, 2018); therefore, 1Cx values are independent of ploidy and can be used to compare the amount of DNA in species with different ploidy levels. Plants with higher ploidy levels are expected to have less DNA in each chromosome than diploids (Sliwinska et al., 2022). As expected, the average genome size of diploid *Pycnanthemum* species had larger 1Cx genomes than the tetraploids and hexaploid. Studies also found positive correlations between chromosome number and genome size in *Crepis* and *Callicarpa* (Contreras & Ruter, 2011; Godelle et al., 1993). However, genome size is not consistently correlated with chromosome number or ploidy levels. Maynard and Ruter (2022) found no correlation between the genome size of *Salvia* and the chromosome number or ploidy level, concluding that ploidy level and chromosome number could not predict the genome size of *Salvia*.

Genome size can also reflect the adaptations of a species to the plant habitat. Larger genomes are slower to adapt to environmental constraints, while smaller genomes enable the plants to respond to unfavorable conditions and cycle through generations quickly (Bennett, 1972; Suda et al., 2015). However, polyploidy and greater chromosome number also confer advantages. Polyploidy introduces heterosis, increasing vigor, and gene redundancy, stabilizing the genome and protecting against lethal mutations (Comai, 2005). The chromosome numbers reported in *Pycnanthemum* ranged from x = 18, 19, or 20 (Chambers, 1961a; Chambers &

Chambers, 1971), which indicates dysploidy or aneuploidy. The loss and gain of chromosome numbers may make phylogeny less obvious and alter compatibility between closely related plants. Identifying connections between the genome size, chromosome number, and ploidy level of *Pycnanthemum* will allow breeders to predict currently unknown genome sizes. Additionally, a strong relationship between ploidy, chromosome number, and genome size can inform breeding programs by allowing breeders to infer compatibility between plants and predict the vigor of progeny based on genome size.

Comparing the Base Pair Composition

The GC% ranged from 29.32% (*P. incanum*) to 34.47% (*P. setosum*). The difference between the PI and DAPI values increased as the difference between the GC content of the sample and the standard increased. Ortega-Ortega et al. (2019) and Parris et al. (2010) reported similar findings, noting an increase in variation between DAPI and PI values as the deviation from the standard GC% increased. The 2C values calculated from DAPI in the study were consistently lower than the PI values, except in *P. setosum*. Previous studies have reported DAPI overestimating and underestimating genome sizes compared to PI. Šiško et al. (2003) found that 2C values run with DAPI were lower than those stained with PI in *Cucurbita*, while Contreras and Shearer (2018) determined that the DNA content of *Acer* stained with DAPI was higher than the DNA content reported by PI. DAPI preferentially binds to AT base pairs, while PI intercalates into the DNA, dying all base pairs (Šmarda & Bureš, 2010). Therefore, DAPI is not an accurate fluorochrome for genome size analysis when the AT:GC ratio of the sample differs from the standard (Parris et al., 2010). The GC content of *Pycnanthemum* species and standards were not significantly different, indicating that DAPI can accurately determine the DNA content of the *Pycnanthemum* species in the study.

The strong relationship between the genome sizes reported in this study and the ploidy levels and chromosome numbers published by Chambers and Chambers (2008) indicates that genome size and ploidy levels can be confidently predicted. Future studies should work to determine the genome sizes of the remaining species and clarify if intraspecific variation exists within *Pycnanthemum*. Genome size is a useful tool for breeding programs, helping to identify germplasm and predict compatibility, which allows for faster and more efficient cultivation. Determining the genome size of *Pycnanthemum* will inform breeding decisions and improve the efficiency of breeding programs.

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Tables and Figures

$$Genome\ size_{unknown} = Genome\ size_{standard} \times \left(\frac{mean\ fluorescence_{unknown}}{mean\ fluorescence_{standard}}\right)$$

Figure 4.1. Calculating an unknown genome size.

$$AT\%=AT\%_{standard} \times \left[\left(\frac{\text{DAPI fluorescence}_{standard}}{\text{DAPI fluorescence}_{unknown}}\right) \div \left(\frac{\text{PI fluorescence}_{standard}}{\text{PI fluorescence}_{unknown}}\right)\right]^{\frac{1}{\text{binding length}}}$$

Figure 4.2. Calculating the AT content of an unknown genome.

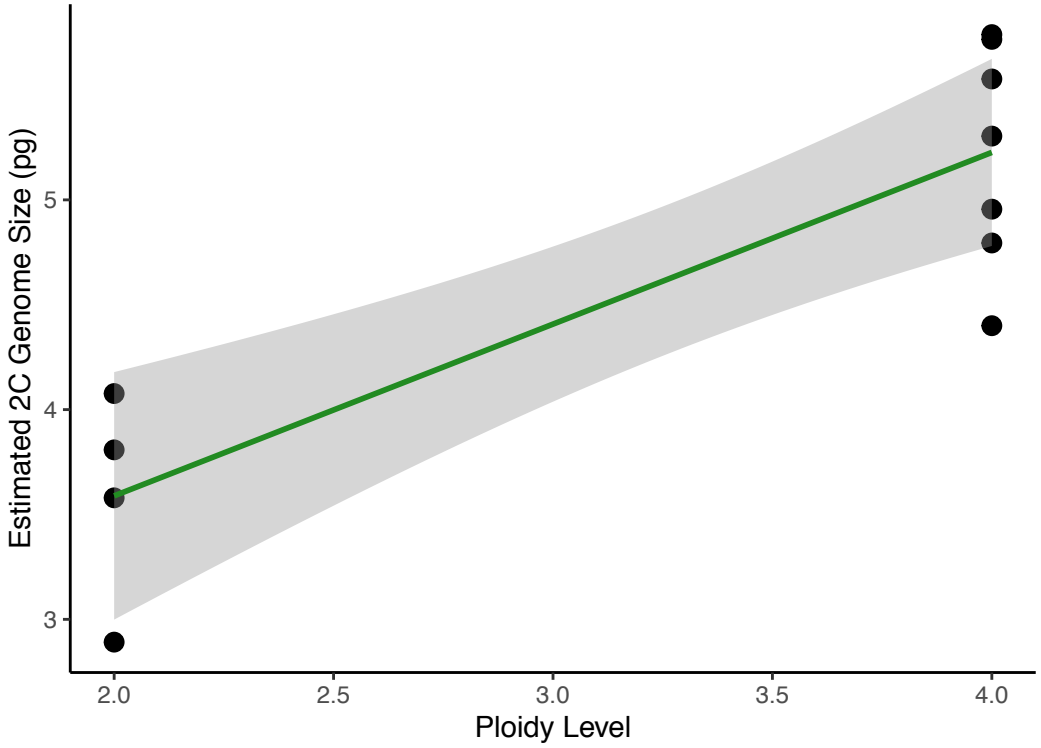

Figure 4.3. The correlation between ploidy levels and 2C values of *Pycnanthemum* species. There was a strong relationship between the ploidy levels proposed by Chambers and Chambers (2008) and the estimated 2C values ($R^2 = 0.70$; $y = 0.8052x + 1.9784$).

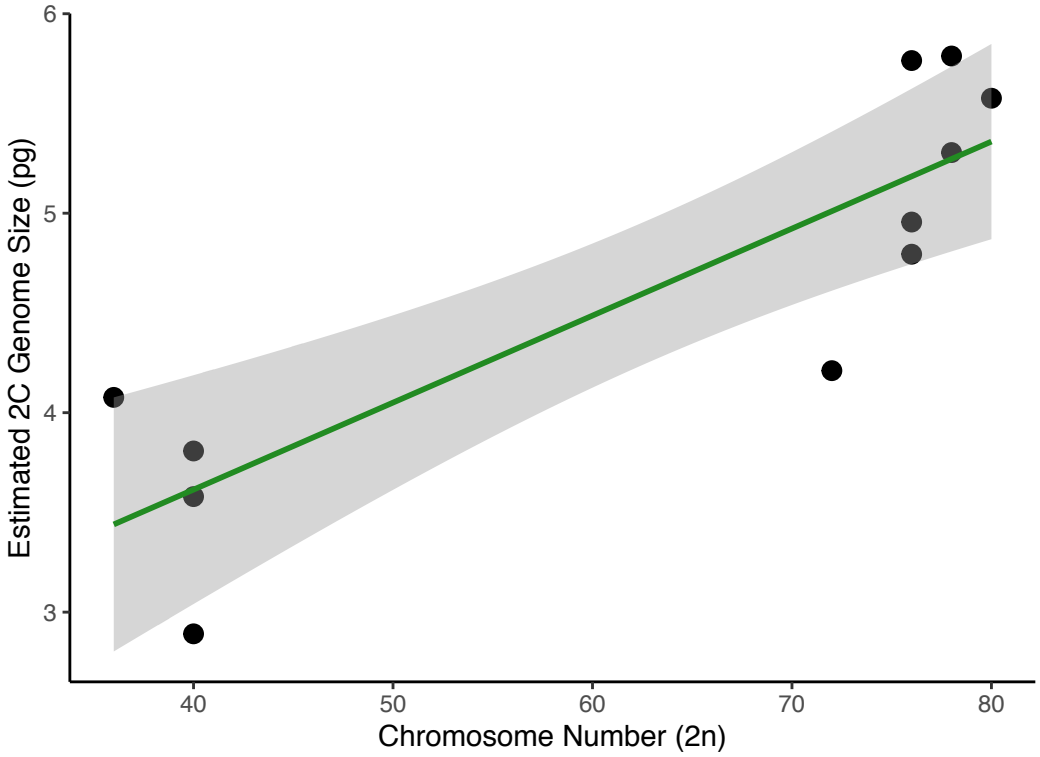

Figure 4.4. The correlation between chromosome numbers and 2C values of *Pycnanthemum* species. The chromosome numbers reported by Chamber and Chamber (2008) were closely related to the estimated genome sizes ($R^2 = 0.74$; $y = 0.0436x + 1.8693$).

Table 4.1. Table detailing the acquisition of the germplasm used in the study.

Genus *Pycnanthemum*	Accession ID	Propagule	Collection Data
P. albescens 'Malcolm Vidrine'	12627	whole plant	Merryville, Louisiana: Almost Eden
P. beadlei	619314	seed	Corvallis, OR: USDA-ARS National Clonal Germplasm Repository
P. californicum	619302	seed	Corvallis, OR: USDA-ARS National Clonal Germplasm Repository
P. curvipes	619336	seed	Corvallis, OR: USDA-ARS National Clonal Germplasm Repository
P. flexuosum	Flex SH	whole plant	Athens, GA: Mimsie Lanier Center for Native Plant Studies
P. floridanum	619281	rhizomes	Corvallis, OR: USDA-ARS National Clonal Germplasm Repository
P. incanum	Inc SH	whole plant	Athens, GA: Mimsie Lanier Center for Native Plant Studies
P. muticum	619319	seed	Corvallis, OR: USDA-ARS National Clonal Germplasm Repository
P. pilosum	Pilo SH	whole plant	Athens, GA: Mimsie Lanier Center for Native Plant Studies
P. pycnanthemoides	619301	seed	Corvallis, OR: USDA-ARS National Clonal Germplasm Repository
P. setosum	619278	rhizomes	Corvallis, OR: USDA-ARS National Clonal Germplasm Repository
P. torreyi	619271	rhizomes	Corvallis, OR: USDA-ARS National Clonal Germplasm Repository
P. virginianum	W657076	seed	Pullman, WA: USDA-ARS Western Regional Plant Introduction Station
P. nudum	DOE3	cuttings	Doerun, GA: Doerun Pitcher plant Bog Wildlife Management Area

Table 4.2. Chromosome numbers, ploidy levels, and estimated genome sizes of *Pycnanthemum* species. The internal standards are listed as either 1 (*Pisum sativum*) or 2 (*Solanum lycopersicum*). The chromosome numbers were determined experimentally by Chambers (1961b) and Chambers and Chambers (1971), while ploidy levels were proposed by Chambers and Hamer (1992) (indicated by asterisks). The 1Cx value of *P. albescens* assumes the accession tested was a diploid, *P. muticum* was a pentaploid, and *P. torreyi* was a hexaploid (ploidy was calculated using the linear equation y = 0.8052x + 1.9784).

Genus *Pycnanthemum*	Estimated 2C genome size ± SD (pg)	Internal Standard	Chromosome Number (2n)	Ploidy Level*	Estimated 1Cx Value (pg)
P. albescens	3.30 ± 0.16	1	38, 76	diploid, tetraploid	1.65
P. beadlei	4.96 ± 0.13	2	76	tetraploid	1.24
P. californicum	2.89 ± 0.08	1	40	diploid	1.45
P. curvipes	3.58 ± 0.16	1	40	diploid	1.79
P. flexuosum	4.08 ± 0.06	1	36	diploid	2.04
P. floridanum	5.30 ± 0.22	2	78	tetraploid	1.33
P. incanum	4.79 ± 0.23	2	76	tetraploid	1.20
P. muticum	5.82 ± 0.21	2	40, 80, 108	diploid, tetraploid, pentaploid	1.16
P. pilosum	5.79 ± 0.26	1	78	tetraploid	1.45
P. pycnanthemoides	4.21 ± 0.09	2	72	tetraploid	1.10
P. setosum	5.77 ± 0.10	2	76	tetraploid	1.44
P. torreyi	7.22 ± 0.24	2	80, 120	tetraploid, hexaploid	1.20
P. virginianum	5.58 ± 0.13	1	80	tetraploid	1.39
P. nudum	3.81 ± 0.08	1	40	diploid	1.90

CONCLUSION

Pycnanthemum is a pollinator plant with market potential, though the novelty of the genus makes initial introduction difficult. Previous studies have compared *Pycnanthemum* to other genera and found that *Pycnanthemum* attracts a greater abundance and variety of pollinators. Therefore, cultivation efforts should focus on improving the market value of the genus to encourage its use as a pollinator plant in suburban or urban landscapes. The public could serve as a great ally in conserving pollinators and mitigating the effects of habitat fragmentation in rural and urban areas. Breeding pollinator plants with landscape traits encourages cities to supplement pollinator habitats with small patches of resources. Determining that *Pycnanthemum flexuosum* attracted the most pollinators in each category (besides wasps) provides a foundation for a breeding program combining pollinator conservation and consumer appeal. Currently, *Pycnanthemum* production procedures have not been the subject of research, which makes introduction and cultivation difficult. To address this uncertainty, *Pycnanthemum* was examined for its response to increasing concentrations of paclobutrazol, a commonly used plant growth regulator (PGR). *Pycnanthemum virginianum* exhibited a decrease in height as paclobutrazol concentrations increased. Drenching plants with 4 mg a.i./pot increased flowering, although density did not differ from the control, while 8 mg a.i./pot treatments increased density but did not affect flowering. *Pycnanthemum flexuosum* should be treated with concentrations lower than 6 mg a.i./pot to maintain flowering. An additional tool for introducing new plants to cultivation is genome size. The DNA content of plants reveals phylogenic information, informs breeding decisions, and improves identification. According to the species tested, the 2C value of

Pycnanthemum can confidently be predicted by chromosome number and previously proposed ploidy levels. Using the estimated genome sizes, breeders can infer compatibility and develop breeding goals. *Pycnanthemum* is a native herbaceous perennial with potential market value that attracts a variety and abundance of pollinators. Exploring cultivation of the genus will inform future breeding and production efforts, encouraging the use of *Pycnanthemum* in landscapes and, subsequently, as supplemental pollinator habitat.

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APPENDIX

APPENDIX A. COMPARISON OF THE EFFECT OF IBA TREATMENTS ON *PYCNANTHEMUM* SPECIES

Introduction

Expediating plant growth and improving success in the greenhouse makes plants, especially new introductions, more accessible to growers and breeders. *Pycnanthemum* would be a relatively new introduction to the market with no production protocols; therefore, growers may be hesitant to work with the genus. Determining the best practices for managing plant care will allow for more efficient plant growth and faster adoption of new plants into growing programs.

Rooting hormones are used by growers to improve rooting and increase the survival of propagules. Indole-3-butyric acid (IBA) is a commonly used rooting hormone available as a liquid or powdered dip. IBA is an ideal hormone for propagation because of its role in lateral and adventitious root development and root hair elongation (Frick & Strader, 2018). Propagules rely on roots to uptake the water and nutrients required for shoot growth; therefore, determining optimal IBA concentrations improves propagation success. The ideal concentration of IBA differs between plants. For example, one study reports that 1500 mg/L IBA increases the density of *Streptosolen jamesonii* root systems (Pêgo et al., 2019). On the other hand, Rehana et al. (2020) found that *Crossandra infundibuliformis* cuttings treated with 3000 mg/L IBA were the most successful. Plants can establish faster when root systems develop quickly, improving greenhouse production speed. However, the plant response to IBA varies between species, meaning optimal IBA concentrations must be determined for each plant.

Plant rooting and survival at IBA concentrations higher than the ideal dose may plateau, causing nonsignificant improvements. However, in some cases, treating cuttings with IBA concentrations that are too high can reduce rooting (Carpenter & Cornell, 1992). Tien et al. (2020) found that *Solanum procumbens* cuttings treated with higher concentrations than the optimum dosage (500 mg/L IBA) reduced shoot growth and root development. When treated with excessive IBA concentrations, fewer plants developed new shoot growth, root systems were less dense, and roots were shorter on average (Tien et al., 2020). The success of propagation decreases when IBA concentrations are not optimal. Applying concentrations of IBA that are lower than ideal limist the development of propagules, while higher concentrations waste resources and harm plant production. Finding the IBA concentration that results in the maximum propagule survival provides growers and breeders with efficient procedures for plant production.

Pycnanthemum virginianum and *Pycnanthemum flexuosum* are members of the *Lamiaceae*, characterized by small, tubular flowers and a minty fragrance when the leaves are bruised. The herbaceous perennials have white or purple flowers with purple speckling. *Pycnanthemum virginianum* is native to the central and eastern U.S., while *P. flexuosum* is native to the southeastern U.S. (Hummer et al., 2020; Weakley, 2020). *Pycnanthemum* is a relatively unexplored genus that attracts pollinators and has the potential to appeal to consumers. Developing production procedures for the genus will make the plant more accessible to growers and aid in breeding programs that aim to improve *Pycnanthemum* for the public market.

Materials and Methods

Plant Material

Pycnanthemum virginianum and *P. flexuosum* seeds (W657076 and W654808, respectively) from the USDA-ARS Western Regional Plant Introduction Station (Pullman, WA)

were grown, then transplanted into 6.65 cm (280 mL) Deep Press Fit Pots (The HC Companies, Twinsburg, GA) of Pro-line C/B Growing Mix (Jolly Gardener, Shady Dale, GA). Plants were grown in a greenhouse set to day/night conditions of 25°C /20 °C with 40% / 30% humidity located at the University of Georgia's Durham Horticulture Farm (33.944507, -83.375774). Fertilization occurred weekly using 200 mg/L of Jack's Professional Water Soluble Fertilizer 20-10-20 Peat-Lite (20-4.4-16.6 N-P-K) (JR Peters Inc., Allentown, PA). The intial plants were maintained in the greenhouse as stock plants.

Compare the effectiveness of powdered and liquid rooting hormone

Pycnanthemum virginianum and *P. flexuosum* were used as stock plants for this study. Ten cuttings were dipped into 0, 1,000, 3,000, and 5,000 mg/L K-IBA for 10 seconds. The propagation solution was a mixture of K-IBA, water-soluble IBA (Kroin, 2017), with water. Another ten cuttings were treated with Hormex Rooting Powder No. 8 (Hormex, Westlake Village, CA), a powdered rooting hormone. Cuttings were trimmed to 3-3.5 cm, then treated. Immediately following treatment, propagules were planted in 6.65 cm (280 mL) pots with a 1:1 ratio of Pro-line C/B Growing Mix (Jolly Gardener, Shady Dale, GA) to perlite and left under mist. The propagation bench sits under a humidity tent with 21°C bottom heat, 70% shade cloth, and mist every five minutes for five seconds during daylight hours. After four months, plant survival was examined.

Statistical Analysis

An ANOVA analysis (RStudio, PBC, Version 1.4.1725, Boston, Massachusetts) was used to determine if the treatments affected plant survival and a Tukey HSD post-hoc test (RStudio) was used to compare survival between treatments. Significance was reported as $p \leq 0.05$.

Results

Treating *Pycnanthemum virginianum*

Treatment with 0, 1,000, 3,000, and 5,000 mg/L IBA and powdered rooting hormone resulted in 90% *P. virginianum* survival across treatments (Table A.1); therefore, there was no correlation between the concentration of liquid IBA and survival ($R^2 = 0$). Rooting and survival did not increase or decrease after using powdered or liquid IBA.

Treating *Pycnanthemum flexuosum*

Few *P. flexuosum* plants treated with 0 mg/L and powdered IBA survived (30%). In contrast, half of plants treated with each concentration of liquid IBA (1,000, 3,000, and 5,000 mg/L IBA) survived treatment (Table A.1). Survival of propagules improved when treated with IBA solution compared to the control and powder. However, there was no significant relationship between increasing liquid IBA concentrations and cutting survival ($R^2 = 0.46$).

Discussion

Pycnanthemum virginianum propagules could be a vigorous species that requires little assistance with rooting, which would explain the lack of response to the IBA treatments tested during the study and the overall success of propagation. The similar response between treated plants and the control indicates that *P. virginianum* can produce sufficient adventitious roots without supplemental IBA. However, the lack of decreasing survival in the study suggests that IBA doses higher than those tested could affect survival. Higher concentrations could improve propagation, although it is also possible that higher concentrations could reduce success.

The survival of *P. flexuosum* did not exceed 50%, though liquid IBA treatments increased survival compared to the control and powdered treatment. Therefore, *P. flexuosum* performs better when treated with liquid IBA than the powdered rooting hormone. Propagule survival did

not vary between liquid IBA treatments, suggesting the optimal dose was not within the range tested. Higher concentrations may confer greater propagation success, though concentrations too high could be damaging. Rooting hormones, such as IBA, encourages rooting to a point (Costa Junior et al., 2018), after which higher concentrations can reduce rooting.

Pycnanthemum virginianum propagules rooted more successfully than *P. flexuosum* . The difference in the success of propagation between the species could be attributed to differing vigor or sensitivity to hormonal changes. The higher survival percentage of *P. virginianum* controls compared to *P. flexuosum* controls could be due to more vigorous rooting. It is also possible that the hormone equilibrium of *P. flexuosum* is more sensitive than *P. virginianum*. Additional IBA could disrupt the plant processes of *P. flexuosum* more intensely than *P. virginianum*. The IBA concentrations higher than the optimal dose can negatively affect root development and, therefore, the formation of new shoot tissue. Excessive IBA applications can inhibit root development by reducing the abundance of root primordia (Carpenter & Cornell, 1992) or disturbing the equilibrium between plant hormones, resulting in harmful IBA levels (Costa Junior et al., 2018). Plant hormones are present in small concentrations in plants, allowing for signaling through the disruption of concentrations. Therefore, excessive hormone concentrations can negatively impact plant processes, affecting root and shoot development. Costa Junior et al. (2018) demonstrated the effects of excessive IBA when the study found that IBA concentrations above 1000 mg/L reduced the shoot dry weight of *Rhaphiodon echinus*. Similarly, *P. virginianum* and *P. flexuosum* may experience a reduction or plateau in survival or rooting as IBA concentrations increase. However, the dose of treatment that causes a reduction in vigor will differ between species.

The maximum percent of rooted cuttings for *P. flexuosum* was 50%, which is not ideal for plant propagation. When only half of plants root, growers must double the production resources while only providing half the material propagated. Therefore, future studies should test additional rates of IBA. Further studies should also explore higher and lower IBA concentrations than those tested in the study to determine the optimal dose of IBA for *P. virginianum* rooting. Developing procedures for plant propagation improves the accessibility of new introductions for growers and breeders. *Pycnanthemum* is a pollinator plant with market potential, though the genus has seen little cultivation and research. Determining ideal IBA dosages will facilitate propagation and encourage more programs to embrace *Pycnanthemum*.

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Tables and Figures

Table A.1. Survival percentages after various IBA treatments in *Pycnanthemum*. *Pycnanthemum* species propagated and examined after four months for survival.

	IBA treatment (mg/L)	Survival (%)
Genus *Pycnanthemum*		
P. virginianum	0	90 a
	1000	90 a
	3000	90 a
	5000	90 a
	Powder	90 a
P. flexuosum	0	30 a
	1000	50 a
	3000	50 a
	5000	50 a
	Powder	30 a

APPENDIX B. EVALUATING VEGETATIVE AND REPRODUCTIVE CHARACTERISTICS OF *PYCNANTHEMUM* SPECIES

Abstract

Breeding programs rely on variability within a population; therefore, establishing an understanding of growth characteristics informs hybridization decisions and selection. Plant identification is also crucial to efficient cultivation, shaping the expectations of growers and consumers. *Pycnanthemum* is a pollinator-attractive plant with potential consumer appeal that could be cultivated for landscape use to encourage the public to participate in pollinator conservation. The current morphology-based keys for *Pycnanthemum* rely on traits that develop later in the plant's life cycle, which complicates identification. The study compared the growth habit (height, growth index, area of the basal crown, and leaf abscission on the lower stem), reproductive traits (flowers per stem and flower color), and vegetative characteristics (leaf shape, leaf margin, leaf color, and pubescence). Most traits did not differ between species, though *P. floridanum*, *P. pilosum*, and *P. verticillatum* were the tallest, while *P. incanum* was the shortest. *Pycnanthemum incanum* also had the darkest green leaves, while *P. virginianum* and *P. californicum* had the lightest green leaves. Describing the vegetative and reproductive characteristics of *Pycnanthemum* species will provide a foundation for breeding and cultivation. Introducing additional traits to morphological keys will also offer additional guidance for species identification.

Introduction

Establishing the extent of phenotypic variability in a population is an integral step in launching new breeding programs. Examining the variety of traits within a genus allows breeders to formulate breeding goals and consider hybridization decisions. Describing the different phenotypes within a genus reveals characteristics to improve and options for introducing the desired modifications from within the population. Diverse traits may also be related to the environmental conditions of a species' native range. Species may have adapted to specific climates or harsh conditions, which could offer stress tolerance or novel traits to a breeding program.

Additionally, understanding the morphology of plant species increases the accuracy of identification. Accurate identification within a genus informs breeders, consumers, and growers about distribution ranges, expected growth habits, ecosystem services, and compatibility. The phylogeny of plant species can also help breeders predict hybridization success, though plants must be correctly identified. Misidentification could reduce the success of crosses, cause consumers to place species where they cannot thrive, and result in the mishandling of plants during production. Therefore, identification could assist with the production and cultivation of plants.

Pycnanthemum (commonly known as mountain mint) is a member of the *Lamiaceae* native to North America. Weakley's key (2020) and Chambers' dissertation on *Pycnanthemum* (1961b) attempt to elucidate species identification, though certain traits are not discussed. Previous keys distinguish *Pycnanthemum* species from each other using several attributes, such as plant height, leaf characteristics, stem pubescence, and calyx traits (Chambers & Hamer, 1992; Chambers, 1961b; Weakley, 2020; Wofford, 1989). Currently, *Pycnanthemum* is separated

into seven sections (*Pycnanthemum*, *Aristatae*, *Brachystemum*, *Capitellatae*, *Macrocephalae*, *Nudae*, *Californicae*) based on the chromosome number, compatibility, and morphology of species (Chambers & Chambers, 2008). Chambers (1961b) and Chambers and Chambers (1971)found a mixture of base numbers (x = 18, 19, 20) and ploidy levels (2n = 2x, 4x, 5x) within *Pycnanthemum*. Chambers and Hamer (1992) also proposed *P. torreyi* could be a hexaploid with x = 20. The compatibility of plants was also observed through hybridization and subsequent pollen staining of progeny (Chambers & Chambers, 1971). The proposed grouping of *Pycnanthemum* species has changed over time, with initial studies grouping plants into the Incanum Phylad, the Virginianum Phylad, and other periphery groups (Grant & Epling, 1943). The difficulty groupings *Pycnanthemum* demonstrates that the species can be challenging to identify.

Examining traits related to plant development also informs production procedures, breeding decisions, and consumers' purchases. Providing information about plant growth eases the introduction of new plants to the market by improving identification and informing expectation of plant charactristics, such as plant dimensions and flower color. Current keys for the genus rely on calyx lobe lengths to differentiate species, which can prevent identification until the plants begin to produce reproductive growth. However, *Pycnanthemum* may not readily bloom in the first year of production (Dr. Svoboda V. Pennisi, personal communication), delaying identification further. Verifying current information and introducing characteristics to distinguish between species could assist with earlier and more accurate identification. Comparing species also assists in breeding programs by helping breeders select superior plants and formulate breeding goals. Breeding programs should aim to improve the floral characteristics,

growth habits, and vegetative traits of *Pycnanthemum.* Characterizing each species will identify areas requiring improvement and provide parameters for breeding programs.

Pycnanthemum beadlei, *P. curvipes*, *P. flexuosum, P. floridanum, P. incanum, P. muticum*, *P. pilosum*, *P. pycnanthemoides*, *P. setosum*, *P. tenuifolium, P. torreyi*, *P. verticillatum*, and *P. virginianum* originate from the central and eastern regions of the U.S., while *P. californicum* is native to California (Hummer et al., 2020). *Pycnanthemum* is an herbaceous perennial that emits a minty or thyme-like fragrance when the leaves are bruised. The flowers are white or purple with purple speckling and arranged in a cyme. Some species have canescent (whitened) leaves (Weakley, 2020), which gives plants a silvery appearance as vegetation develops.

Pycnanthemum is a pollinator-attractive plant (MacLeod et al., 2020; Porter, 2010) with market potential, which could encourage the public to participate in pollinator conservation. *Pycnanthemum* would be a useful addition to landscapes and gardens as a link connecting pollinator populations and habitats to mitigate the effects of habitat fragmentation on pollinator populations (Menz et al., 2011). The genus attracts an abundance of pollinators, as Porter (2010) demonstrated when more beneficial insects were observed visiting *P. flexuosum* compared to other genera. In addition to pollinator abundance, *Pycnanthemum* also supports species richness. Harris et al. (2022) reported that *Pycnanthemum verticillatum var. pilosum* attracted 19 pollinator families. Consumers would appreciate *Pycnanthemum* for its pleasant fragrance, flowers, and potential adaptability. *Pycnanthemum* species span North America and are acclimated to various climates, making the plant more tolerant of environmental stress (Thomas & Schrock, 2004). Additionally, the genus appeals to the native plant market as a native perennial with ornamental value (Jenderek et al., 2013). *Pycnanthemum* attracts pollinators and

expresses traits with market appeal. The genus would be a promising candidate for cultivation and breeding aimed towards landscape use and pollinator support.

Materials and Methods

Plant Material and Plot Arrangement

Fourteen species of *Pycnanthemum*, including *P. beadlei*, *P. curvipes*, *P. californicum, P. flexuosum, P. floridanum, P. incanum, P. muticum*, *P. pilosum*, *P. pycnanthemoides*, *P. setosum*, *P. tenuifolium, P. torreyi*, *P. verticillatum*, and *P. virginianum,* were planted at the University of Georgia's Durham Horticulture Farm (33.944507, -83.375774). This site is a 90-acre farm located south of the University of Georgia and composed of Cecil sandy loam (USDA). *Pycnanthemum albescens* was also planted but did not perennialize.

Seed procured from nurseries and germplasm repositories (germplasm acquisition is listed in Table B.1.) were sown in a greenhouse with day/night temperatures maintained at 25°C /20 °C and 40% / 30% humidity, then transplanted into 6.65 cm (280 mL) Deep Press Fit Pots (The HC Companies, Twinsburg, GA) in Pro-line C/B Growing Mix (Shady Dale, GA). Seedlings were transplanted into 6.65 cm (280 mL) pots and placed on a propagation bench with 70% shade, 21°C bottom heat and misted every five minutes for five seconds. The initial germplasm was grown as stock plants. Ten cuttings were taken from each of the fourteen stock plants and treated with 3,000 mg/L Indole-3-butyric acid (IBA) solution, then placed in 6.65 cm (280 mL) pots with a 1:1 ratio of Pro-line C/B Growing Mix (Shady Dale, GA) to perlite. Once plants developed roots within the 6.65 cm (280 mL) pots, they were transplanted into C300 2.8 L pots (Nursery Supplies Inc., Kissimmee, FL) with Pro-line C/B Growing Mix and 24 g of Osmocote Plus 15-9-12, 8-9-month (15-4-10 N-P-K). Plants were grown in the greenhouse and fertilized with 200 mg/L of Jack's Professional Water each week. After establishing roots in

C300 2.8 L pots, plants were transplanted into the ground during the spring and summer of 2022. The plants were arranged in randomized complete blocks spaced 1 m apart. A 20 mm wide drip tape line with emitters spaced 30 cm apart was sufficient for inground plants (Rivulis Irrigation Ltd., San Diego, CA). Irrigation ran for 90 minutes four times a week. Pine straw was laid between the plants as mulch, and plots were hand-weeded to combat weed pressure.

Data Collection

The growth habits, flowering characteristics, and vegetative traits of three plants per species were measured and averaged to provide a profile for each plant. The parameters of growth habit include the height, growth index, area of the basal crown, and leaf abscission on the lower stem. The height of three stems per plant were measured to determine the height. The growth index of the plant was calculated by averaging the height, width 1, and width 2 (perpendicular to width 1). The basal crown area was measured by finding the elliptical area of the crown ($A = \pi$ (1/2 width 1) * (1/2 width 2)). The leaf abscission on the lower portion of the plant was calculated as a proportion of the bare stem to the total stem length. The abscission of leaves was measured on three different stems to determine the average abscission per plant.

The metrics for flower characteristics include flowers per stem and flower color. Flowers per stem were averaged by counting the flower heads on three stems per plant. The flower color was described as purple or white and speckled or not.

The leaf shape, leaf margin, leaf color, and pubescence were noted to compare the vegetative traits of individual plants. Leaf shape and margin were recorded according to the morphology specified by the UF/IFAS Center for Aquatic and Invasive Plants (2009) (Figure B.1). Leaf color was determined using a Minolta SPAD-502 Chlorophyll Meter (Spectrum Technologies Inc., Plainfield, IL). The SPAD (Soil-Plant Analysis Development) readings of

three leaves per plant were averaged to calculate the mean greenness per plant. Pubescence on leaves was also noted on the upper and lower leaf surface.

Pycnanthemum californicum and *P. curvipes* were introduced to the plot during the spring of 2023; therefore, data involving growth habit was not collected on either species. Additionally, *P. californicum* did not flower, therefore, only traits related to leaves were collected.

Statistical Analysis

An ANOVA (RStudio, PBC, Version 1.4.1725, Boston, Massachusetts) was used to determine if the species of *Pycnanthemum* affected growth habit, flowering characteristics, and vegetative traits. The number of flowers per stem was analyzed using a Poisson regression (RStudio)(O'Hara & Kotze, 2010). A Tukey HSD post-hoc test (RStudio) was used to compare the species' growth habits and vegetative traits. A linear regression (Microsoft Excel, Version 16.54, Microsoft Corporation) was also run to determine if a relationship between plant traits and either chromosome number or proposed ploidy level existed. The linear regression was run using only species with one ploidy level. Significance was reported as $p \leq 0.05$.

Results

The tallest plants on average were *P. floridanum, P. pilosum,* and *P. verticillatum* (1.2, 1.1, and 1.1 m, respectively), while *P. incanum* was the shortest (0.6 m). No difference existed in the growth index, average crown area, or the number of flowers per stem between species. The percent of leaf abscission was also not significantly different among species. The average SPAD readings were highest in *P. incanum* (57.2), while *P. virginianum* and *P. californicum* had the lowest SPAD readings (38 and 37.6, respectively). Plant traits were not correlated to the species' chromosome number or proposed ploidy level ($R^2 < 0.45$). The qualitative and quantitative

characteristics of each species are listed in Table B.2 – B.3. The leaves of each of the fourteen *Pycnanthemum* species are depicted in Figure B.2. Additionally, the flowers of thirteen species are shown in Figure B.3.

Discussion

Plant dimensions are critical aspects of breeding programs that determine the target market of the plant. *Pycnanthemum pilosum* was the tallest plant compared to other species in the study. A large plant would fit in native gardens and support pollinator populations. A study comparing wild-type and cultivated *Aronia melanocarpa* found *Andrenidae* preferred native plants despite similar bloom period, due to the native being double the height of the cultivars (Ricker et al., 2019). Similarly, Baker et al. (2020) found monarchs were unable to locate *Asclepias* when tall grasses obscured the plant, though they continued to visit visible milkweed. Some pollinators rely on visual cues to initially locate plants and, once in close proximity, are able to use olfactory signals. Barragán-Fonseca et al. (2020) describes that *Pieris* and *Vanessa* butterflies use color as a visual signal to locate flowers from a distance, and then volatile compounds act as a secondary cue closer to the plants. Pollinator-focused landscapes would benefit from the larger *P. pilosum* plants, though consumers in urban landscapes prefer compact plants.

Traits such as pubescence and volatile compounds can reveal potential defenses against biotic stress, which are desirable traits for cultivation. Most of the *Pycnanthemum* in the study have some level of leaf pubescence, which could indicate the species are better able to defend against herbivory. A study comparing members of the *Lamiaceae* found less spider mite damage and slower pest development on *Salvia officinalis*, which also had the most trichomes (Golan et al., 2021). The increase in trichomes deterred feeding by making contact with the leaf physically

difficult and chemically unpleasant (Golan et al., 2021). Resistance to herbivory is an ideal plant trait for plant breeders and growers cultivating plants for landscapes; therefore, detailing traits that potentially reduce biotic stressors inform plant selection.

Additionally, the *Pycnanthemum* in the study did not appeared to be of interest to deer. Deer tracks were identified throughout the plot multiple times and deer feeding damaged *Ginkgo biloba* and *Ilex creanata* seedlings in an adjacent plot. Despite the presecence of deer no herbivory was found on *Pycnanthemum*. However, Hill (2007) reported that deer significantly damaged *P. torreyi*. Hill (2007) may have obtained an accession of *P. torreyi* that was less deer resistant, or deer populations may have been more desperate in the area during the study conducted by Hill (2007).

Plant health affects consumer perceptions of plants on the market. SPAD values can indicate plant health and attractiveness by measuring leaf greenness. The average SPAD readings were highest in *P. incanum*; therefore, leaves were greener. Relative chlorophyll levels or greenness can be compared between species using SPAD measurements (Castelli & Contillo, 2009), with higher SPAD measurements indicating deeper shades of green. A study comparing the leaf greenness of *Origanum microphyllum* treated with foliar fertilization or root fertilization found root fertilization increased SPAD values, increasing leaf greenness and the aesthetic value (Fanourakis et al., 2022). *Pycnanthemum incanum* may have also had a higher SPAD value than other species because the plants had higher concentrations of macronutrients, influencing chlorophyll activity. Roosta and Arabpour (2013) examined the growth characteristics of *Ocimum basilicum* grown hydroponically and aquaponically. The *O. basilicum* grown in a hydroponic system were larger, denser, and had higher SPAD readings (Roosta & Arabpour, 2013). The study found that hydroponically grown plants had higher concentrations of nitrogen

and manganese, which Roosta and Arabpour (2013) suggest caused the increase in SPAD levels. Nitrogen and manganese are necessary for the structure and function of chlorophyll, which contributes to leaf greenness. SPAD levels indicate the greenness of plants and, in some plants, the relative levels of macronutrients; therefore, plants should be selected to increase greenness.

There was no relationship between the growth traits examined in the study and the proposed ploidy levels of *Pycnanthemum* ($R^2 < 0.4$). Polyploidy can increase the size of plant structures from the cellular level up to plant organs (Madlung, 2013); therefore, the *Pycnanthemum* in the study was expected to display an increase in some aspects of development. *Salvia officinalis* tetraploids increased in height compared to diploids following treatment with colchicine (a doubling agent) (Hassanzadeh et al., 2020). *Thymus persicus* tetraploids also exhibited altered growth compared to diploids, producing darker leaves after colchicine treatments (Tavan et al., 2015). Unlike the previously mentioned plants, *Pycnanthemum* did not display morphological differences in the parameters measured. Other morphology may correlate with polyploidy, such as flower size or leaf thickness, as seen in *Salvia coccinea* and *Salvia patens* cultivars (Kobayashi et al., 2008). Shariat and Sefidkon (2021) also report that the essential oil yield increased in *Satureja khuzistanica* due to polyploid induction. *Pycnanthemum* may have expressed polyploidy in structural and chemical aspects not explored during the study.

Determining the current state of a species improves identification and allows breeders to understand which traits to cultivate for the target market. *Pycnanthemum* differed in height and leaf greenness between species, providing a baseline for selection. Cultivating *Pycnanthemum* for pollinators and consumers requires a balance between pollinator-supportive traits and marketable characteristics. Native perennial pollinator gardens expect different growth habits than urban landscapes; therefore, breeders must consider breeding objectives. *Pycnanthemum* has

potential to appeal to consumers and help the public participate in pollinator conservation. However, *Pycnanthemum* must be selected for pollinator support along with consumer traits to ensure pollinator-attractiveness is maintained. Finding areas of overlap between consumer traits and characteristics that support pollinators will be crucial to developing the genus.

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Tables and Figures

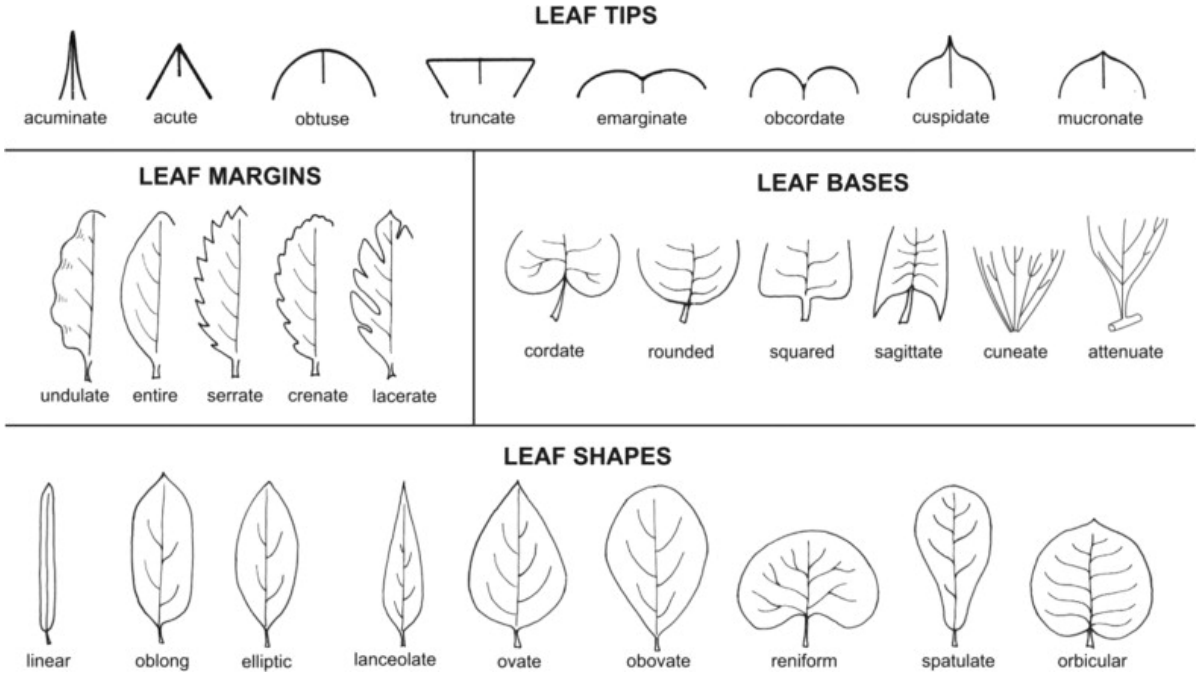

Figure B.1. Depiction of leaf morphology classifications. Figure created by UF/IFAS Center for Aquatic and Invasive Plants (2009)

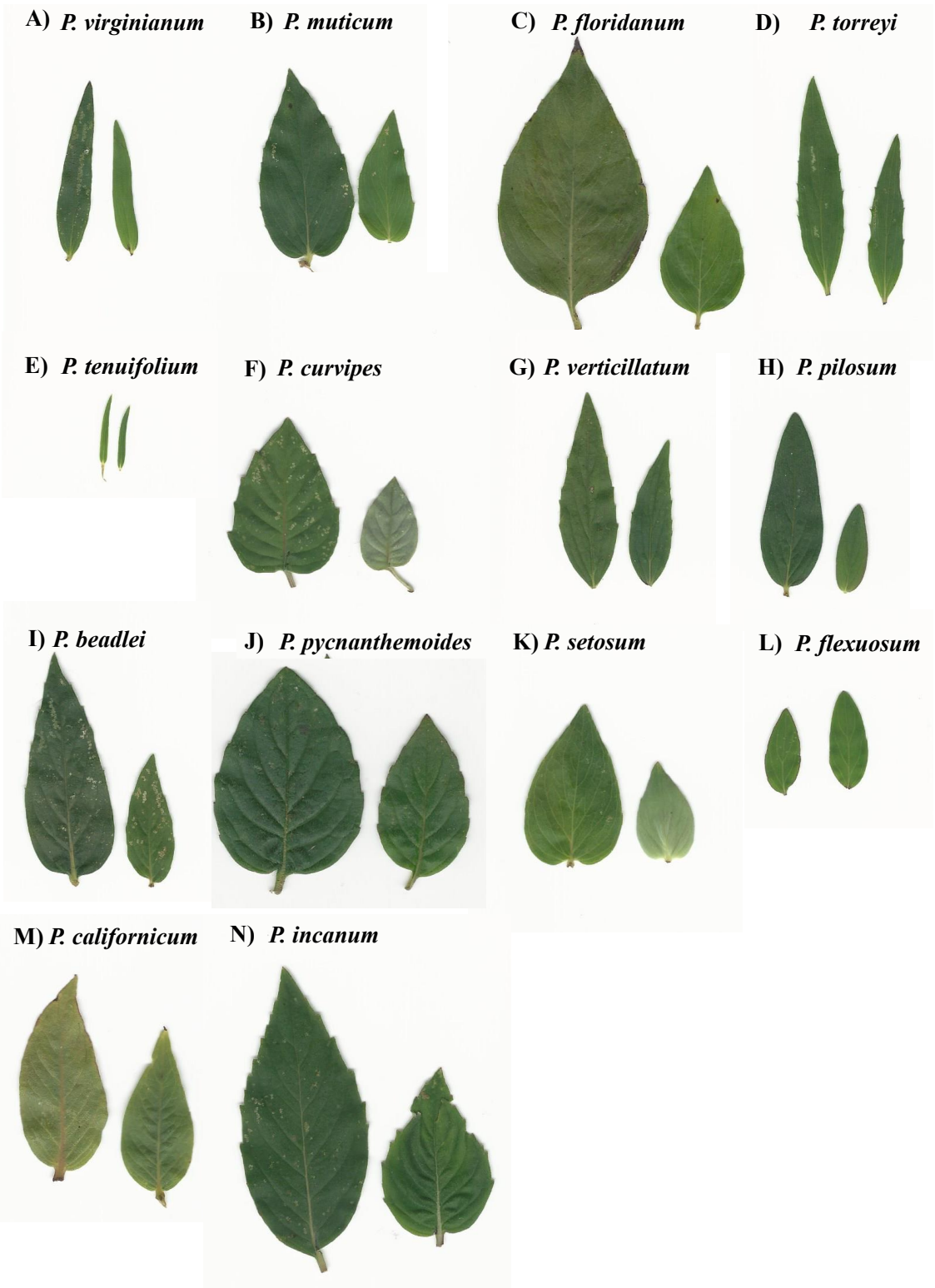

Figure B.2. Leaves of *Pycnanthemum*. On the left is a more mature leaf, on the right is a younger leaf; (A) *P. virginianum*, (B) *P. muticum*, (C) *P. floridanum*, (D) *P. torreyi*, (E) *P. tenuifolium*, (F) *P. curvipes* , (G) *P. verticillatum*, (H) *P. pilosum*, (I) *P. beadlei*, (J) *P. pycnanthemoides*, (K) *P. setosum*, (L) *P. flexuosum*, (M) *P.californicum*, and (N) *P. incanum*.

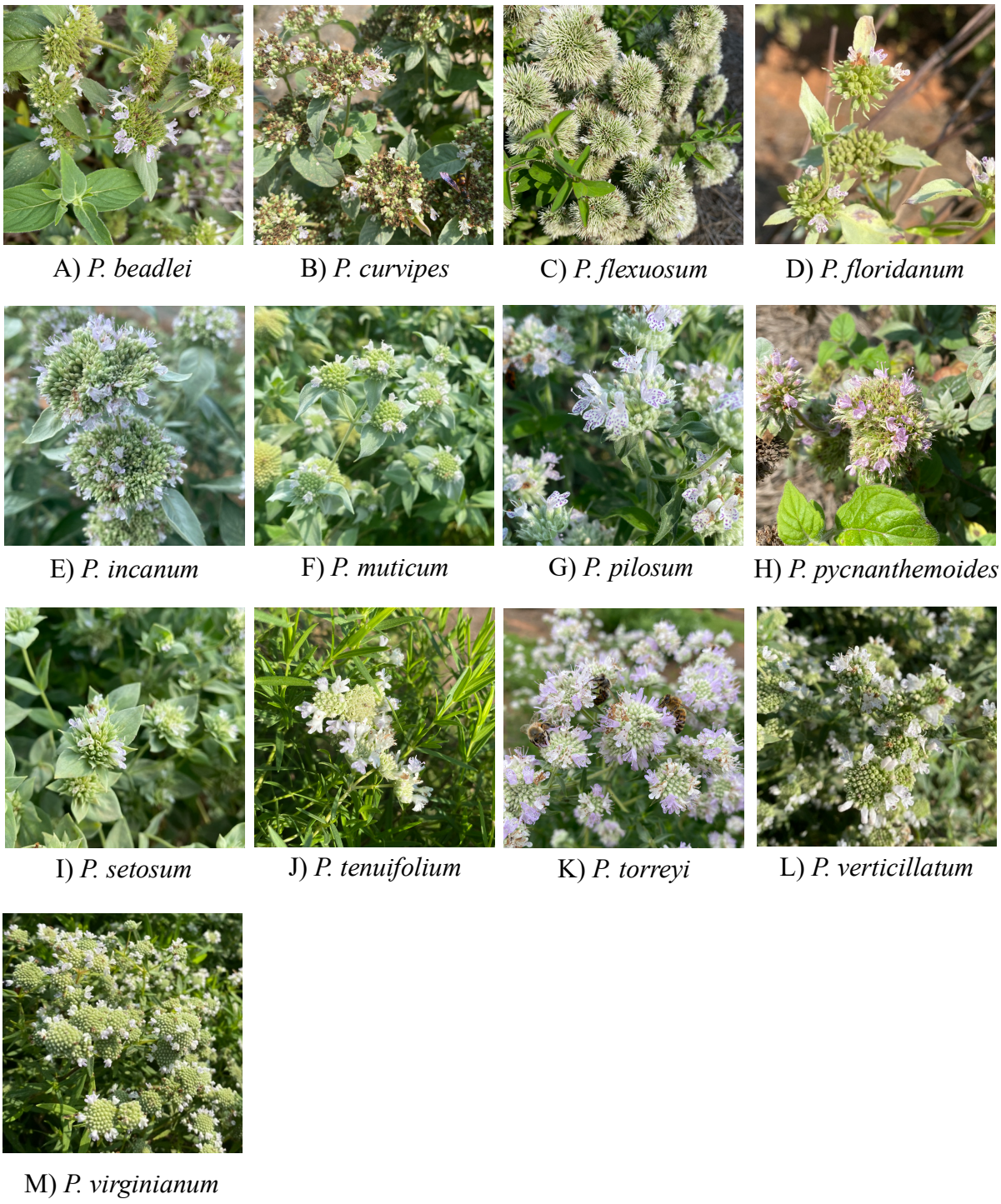

Figure B.3. Inflorescences of *Pycnanthemum*. (A) *P. beadlei*, (B) *P. curvipes,* (C) *P. flexuosum,* (D) *P. floridanum,* (E) *P. incanum,* (F) *P. muticum*, (G) *P. pilosum*, (H) *P. pycnanthemoides*, (I) *P. setosum*, (J) *P. tenuifolium,* (K) *P. torreyi*, (L) *P. verticillatum*, and (M) *P. virginianum*.

Table B.1. Table detailing the acquisition of germplasm used for the study.

Genus *Pycnanthemum*	Accession ID	Propagule	Collection Data
P. beadlei	619314	seed	Corvallis, OR: USDA-ARS National Clonal Germplasm Repository
P. californicum	619302	seed	Corvallis, Oregon: USDA-ARS National Clonal Germplasm Repository
P. curvipes	619336	seed	Corvallis, Oregon: USDA-ARS National Clonal Germplasm Repository
P. flexuosum	Flex SH	whole plant	Athens, GA: Mimsie Lanier Center for Native Plant Studies
P. floridanum	619281	rhizomes	Corvallis, OR: USDA-ARS National Clonal Germplasm Repository
P. incanum	Inc SH	whole plant	Athens, GA: Mimsie Lanier Center for Native Plant Studies
P. muticum	619319	seed	Corvallis, OR: USDA-ARS National Clonal Germplasm Repository
P. pilosum	Pilo SH	whole plant	Athens, GA: Mimsie Lanier Center for Native Plant Studies
P. pycnanthemoides	619301	seed	Corvallis, OR: USDA-ARS National Clonal Germplasm Repository
P. setosum	619278	rhizomes	Corvallis, OR: USDA-ARS National Clonal Germplasm Repository
P. tenuifolium	619332	seed	Corvallis, OR: USDA-ARS National Clonal Germplasm Repository
P. torreyi	619271	rhizomes	Corvallis, OR: USDA-ARS National Clonal Germplasm Repository
P. verticillatum	PYC02F	seed	Winona, MN: Prairie Moon Nursery
P. virginianum	W657076	seed	Pullman, WA: USDA-ARS Western Regional Plant Introduction Station

Table B.2. The qualitative characteristics of *Pycnanthemum* examined in the study.

Genus *Pycnanthemum*	Flower color	Speckling	Pubescence	Leaf margin	Leaf shape
P. beadlei	Light purple to white	Present	Upper and lower surface	Serrate	Lanceolate
P. californicum			None	Entire	Lanceolate
P. curvipes	White	Present	Upper and lower surface	Slightly serrate	Ovate
P. flexuosum	White	Present	Upper and lower surface	Entire	Lanceolate
P. floridanum	White	Present	Lower surface	Slightly serrate	Ovate
P. incanum	Purple to white	Present	Upper and lower surface	Serrate	Lanceolate
P. muticum	White	Present	Veins of lower surface	Slightly serrate	Ovate to lanceolate
P. pilosum	White	Present	Lower surface	Entire	Lanceolate
P. pycnanthemoides	Purple	Present	Upper and lower surface	Serrate	Ovate to lanceolate
P. setosum	White	Present	Velvety but no visible hairs	Serrate	Lanceolate
P. tenuifolium	White	Absent	None	Entire	Linear/Lanceolate
P. torreyi	Purple to white	Present	Lower surface	Entire	Lanceolate
P. verticillatum	White	Present	Upper and lower surface	Slightly serrate	Lanceolate
P. virginianum	White	Present	None	Entire	Lanceolate

Table B.3. The quantitative characteristics of *Pycnanthemum* examined in the study. The height of three stems per plant were measured to determine the height. The growth index of the plant was calculated by averaging the height, width 1, and width 2 (perpendicular to width 1). The basal crown area was measured by finding the elliptical area of the crown (A = π (1/2 width 1) * (1/2 width 2)). The leaf abscission on the lower portion of the plant was calculated as a proportion of the bare stem to the total stem length. The abscission of leaves was measured on three different stems to determine the average abscission per plant. Flowers per stem were averaged by counting the flower heads on three stems per plant.

Genus *Pycnanthemum*	Height (m)	SPAD	Abscission (%)	Basal crown area (cm^2)	Flowers per stem	Growth Index (m)
P. beadlei	0.6 – 1 abc	46.0 abc	18.9 a	561.0 a	41.3 a	1.0 a
P. californicum		37.6 a				
P. curvipes		50.1 cd				
P. flexuosum	0.6 – 0.7 ab	38.6 ab	13.1 a	164.9 a	31.3 a	0.7 a
P. floridanum	1.1 – 1.3 c	43.1 abc	45.0 a	709.7 a	27.6 a	1.2 a
P. incanum	0.5 – 0.6 a	57.2 d	19.2 a	583.0 a	30.3 a	0.9 a
P. muticum	0.8 – 1.1 abc	45.0 abc	27.6 a	1099.0 a	38.3 a	1.2 a
P. pilosum	1.1 – 1.2 c	42.8 abc	23.1 a	2882.1 a	69.8 a	1.5 a
P. pycnanthemoides		46.4 abc				
P. setosum	0.7 – 0.9 abc	45.8 abc	23.8 a	367.0 a	64.0 a	1.0 a
P. tenuifolium	0.6 – 1 abc	38.9 ab	51.6 a	685.7 a	119.7 a	0.8 a
P. torreyi	1 – 1.1 bc	45.6 abc	24.2 a	1134.6 a	51.4 a	1.3 a
P. verticillatum	0.8 – 1.4 c	47.2 bc	26.7 a	2147.0 a	86.1 a	1.3 a
P. virginianum	0.8 – 1.1 abc	38.0 a	26.9 a	628.1 a	86.7 a	1.1 a

APPENDIX C. DETERMINING THE LD_{50} DOSAGE OF EMS AND IRRADIATION (^{60}C) FOR *PYCNANTHEMUM VIRGINIANUM*

Introduction

Genetic variability is the foundation of a breeding program. Traditional breeding programs depend on the genetics inherent in a genus; however, some genera have limited variation. Mutagenesis offers additional genetic variability and introduces unique characteristics by altering qualitative and quantitative traits. Induced mutations can cause silent mutations, lethal mutations, or phenotypic changes. The result of mutagenesis depends on the concentration and exposure period impact the treatment's effect, with the optimal mutagen treatment being the LD_{50}. The LD_{50} is the dose that causes lethal mutations for 50% of the population and provides the highest likelihood of mutation among survivors (Raina et al., 2018). The LD_{50} dose of a mutagen varies with species and mutagen, requiring extensive testing for each plant species.

Ethyl methanesulfonate (EMS) is a chemical mutagen commonly used in breeding programs to induce mutations. The mutagen alters plant material by alkylating nucleotides (Sega, 1984), mainly resulting in the transition of GC pairs to AT pairs (Yan et al., 2021). EMS treatment can affect floral traits, vegetative characteristics, and growth habits. Previous studies have reported EMS treatments increasing *Chrysanthemum indicum* leaf size (Purente et al., 2020), changing the floral structure of *Gossypium hirsutum* (Lian et al., 2020), and causing foliar variegation in *Capsicum annuum* (Siddique et al., 2020). EMS also affects characteristics beyond aesthetic traits, such as increased salt tolerance in *Petunia* (Krupa-Małkiewicz et al., 2017).

Irradiation is another method used to induce mutations, affecting growth characteristics and reproductive traits (Oates et al., 2013). Gamma rays can oxidize amino acids, alter protein conformations, produce free radicals, and destroy covalent bonds, which alters protein structures (Lee et al., 2005) and affects gene expression. An assortment of plant tissue can be treated with irradiation, including seeds, seedlings, cuttings, bulbs, and rhizomes (Datta, 2009). When treating material with irradiation, the dose depends on the exposure length and the irradiation source's age. As the radiation source ages, plant material must be exposed for longer to gain higher concentrations of irradiation. Saika and Jin Hee (2020) suggest that plant breeding goals are generally achievable with low irradiation doses (less than 1 kGy), while higher doses are used for food sterilization. Irradiation can affect a variety of growth characteristics and reproductive traits. Previous studies have found that irradiation improved the chlorophyll content of *Musa spp.* leaves (Abdulhafiz et al., 2018), decreased *Ocimum basilicum* height (Shala, 2019), and introduced novel flower traits in *Rudbeckia subtomentosa* (Oates et al., 2013).

Pycnanthemum virginianum is an herbaceous perennial native to the central and eastern U.S. (Chambers, 1961b). The species has small, tubular white or purple flowers with purple speckling and a minty fragrance, characteristic of other *Lamiaceae* members. Little phenotypic variability exists between *P. virginianum* plants. Mutagenesis could introduce unique traits to the breeding material, accelerating the cultivation of *Pycnanthemum*. *Pycnanthemum virginianum* is a pollinator-attractive plant with market potential. Introducing novel characteristics through mutation will assist cultivation efforts and help the plant appeal to consumers.

Materials and Methods

Plant Material

P. virginianum seeds (W657076) were procured from the USDA-ARS Western Regional Plant Introduction Station in Corvallis, OR. Once seeds germinated, they were transplanted into 6.65 cm (280 mL) Deep Press Fit Pots (The HC Companies, Twinsburg, GA) and used as stock plants for cuttings in a greenhouse with day/night conditions set to 25°C /20 °C and 40% / 30% humidity. Plants were grown in Pro-line C/B Growing Mix (Jolly Gardener, Shady Dale, GA) and initially fertilized with 4 g of Osmocote Plus 15-9-12, 8-9-month (15-4-10 N-P-K) (ICL Specialty Fertilizers, Summerville, SC). Subsequently, plants were fertilized each week with 200 mg/L of Jack's Professional Water Soluble Fertilizer 20-10-20 Peat-Lite (20-4.4-16.6 N-P-K) (JR Peters Inc., Allentown, PA). Root trimming and pruning were used to maintain the plants in the 6.65 cm (280 mL) pots.

Introduce variability into the breeding population using EMS

A preliminary test using 0, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5, and 0.6% concentrations of EMS was used to narrow the LD_{50} range. Treatments were prepared by mixing EMS with deionized water and 0.1% Sil Energy non-ionic surfactant (Brewer International, Vero Beach, FL). The non-ionic surfactant ensured that plant material was thoroughly exposed to the mutating agent. Eight cuttings, taken from *P. virginianum* stock plants, were soaked in each prepared mixture for 6 hours, and constantly mixed at 2.5 Hz on a shaker (New Brunswick Scientific, Edison, New Jersey). Once cuttings were soaked, they were rinsed with water and propagated into liner trays with a 1:1 ratio of Pro-line C/B Growing Mix (Shady Dale, GA) to perlite, then placed under mist. Plants were misted every five minutes for five seconds under 70% shade cloth

and 21°C bottom heat. After nine weeks, the number of surviving propagules were counted to determine the LD_{50} concentration.

Based on the preliminary study results, a second experiment treated cuttings with 0, 0.2, and 0.3% concentrations of EMS. Thirty-two propagules were prepared per treatment using the method described previously. Replicates were arranged in four randomized complete blocks within a single tray with eight samples per replicate. After nine weeks, plants were examined for percent survival.

Introduce variability into the breeding population using gamma irradiation

Irradiation of rooted cuttings occurred at 0, 10, 20, 30, 50, 70, and 90Gy using a Gammacell 200 with a ^{60}Co source (Atomic Energy of Canada, Ottawa, Ontario, Canada) in the Center for Applied Isotope Studies (Athens, GA). Five cuttings per treatment were taken from *P. virginianum* stock plants. Gamma irradiation reduces rooting (Abdulhafiz et al., 2018); therefore, cuttings were planted into 6.65 cm (280 mL) pots with a 1:1 ratio of Pro-line C/B Growing Mix (Shady Dale, GA) to perlite and allowed to root for four weeks before treatment. The number of surviving propagules was counted 12 weeks after treatment to determine the LD_{50} dosage.

Statistical Analysis

A regression analysis was used to determine the predictive equation of survival rates at different concentrations of EMS and irradiation (RStudio, PBC, Version 1.4.1725, Boston, Massachusetts).

Results and Discussion

EMS treatments

The preliminary EMS experiment revealed that 0.5 and 0.6% EMS concentrations were too high, resulting in 0% survival. Of the plants treated with 0.3 and 0.4% EMS, 25% of

propagules survived, while 62.5% survived 0.15 and 0.2% treatments. Therefore, the next step was to test the effect of 0.2 and 0.3% EMS on plant survival.

On average, 87.5% of plants survived the control treatment. The average survival of plants treated with 0.2% EMS was 32.1%, while 0.3% treatments resulted in 17.8% survival. No dominant mutations were observed in the M_1 population. EMS induces mutation by affecting nucleotides (Yan et al., 2021), which modifies the genomic sequence of plants. Therefore, plants treated with low levels of the chemical mutagen are likely to either be unaffected by treatment or have silent modifications. Contrarily, high EMS concentrations are more likely to be mutated and cause lethal mutations, demonstrated by the strong negative relationship between EMS concentrations and *P. virginianum* survival ($y = 572.92x^2 - 411.46x + 87.5$; $R^2 = 0.8882$) (Figure C.1). As the abundance of mutations increases, plant development is more likely to be negatively affected, resulting in plant death. Therefore, lower survival of plants potentially indicates a greater number of mutations, which could provide unique characteristics to a breeding program.

Irradiation Treatments

All plants treated with 10Gy and 20Gy survived, while 30Gy resulted in 83.3% survival. Of the plants treated with 50Gy, 80% survived, 30% survived 70Gy treatment, and 0% survived 90Gy irradiation. As the dose of irradiation increased, plant survival decreased ($y = -0.0134x^2 + 0.058x + 100.66$; $R^2 = 0.9736$) (Figure C.2). Irradiation is a physical mutagen that alters protein structures through amino acid oxidation, destruction of covalent bonds, and free radical production (Piri et al., 2011). Increasing the irradiation dose increases the likelihood of mutation, making the plant more vulnerable to lethal mutations. Therefore, decreasing survival can indicate greater mutation potential. The plants treated in this study tended to survive initial treatments, though the cuttings failed to produce new growth, then yellowed and died weeks after treatment.

Irradiation treatments can affect plants' chemical composition and morphological characteristics (Oates et al., 2013), which could result in physiological and physical modifications. The mutations caused by irradiation can introduce novel traits or negatively impact plant processes, reducing vigor, weakening the plant, and resulting in lower survival.

The survival rates determined in this study suggest the LD_{50} lies within the range of 0.10–0.15% EMS and 55 – 70Gy (Figure D.1 and D.2). Therefore, future studies should perform further EMS treatments between 0.10 – 0.15% EMS and 55 – 70Gy. Studies should also grow plants to maturity to determine which dosages confer dominant mutation more frequently. Additionally, selfing mutated plants would allow researchers to examine recessive mutation among treatments, offering further variability within the breeding population. Breeding programs rely on genetic variability to produce unique plants; however, natural mutation is rare, occurring about 10−5 to 10−8 per loci (Jiang & Ramachandran, 2010). Therefore, induced mutations can efficiently increase the introduction of new traits without introducing DNA from other organisms. The cultivation of *Pycnanthemum* would benefit from greater variability, which could produce novel traits that are appealing to the public.

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Tables and Figures

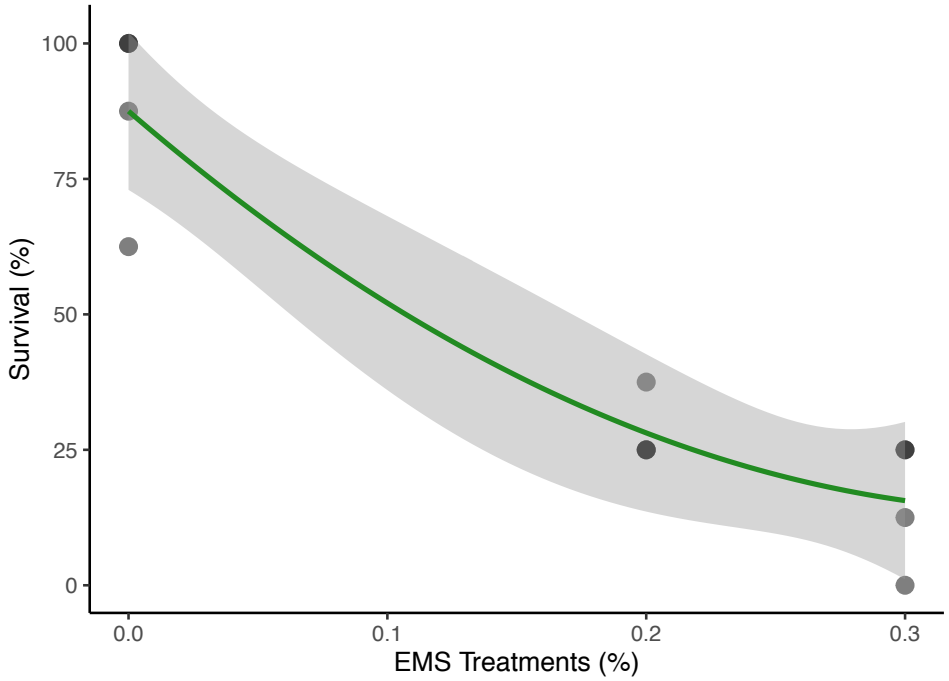

Figure C.1. The survival of *P.* virginianum at different EMS concentrations. The regression equation ($y = 572.92x^2 - 411.46x + 87.5$; $R^2 = 0.8882$) predicts an LD_{50} rate at about 0.11% EMS for 6 hours.

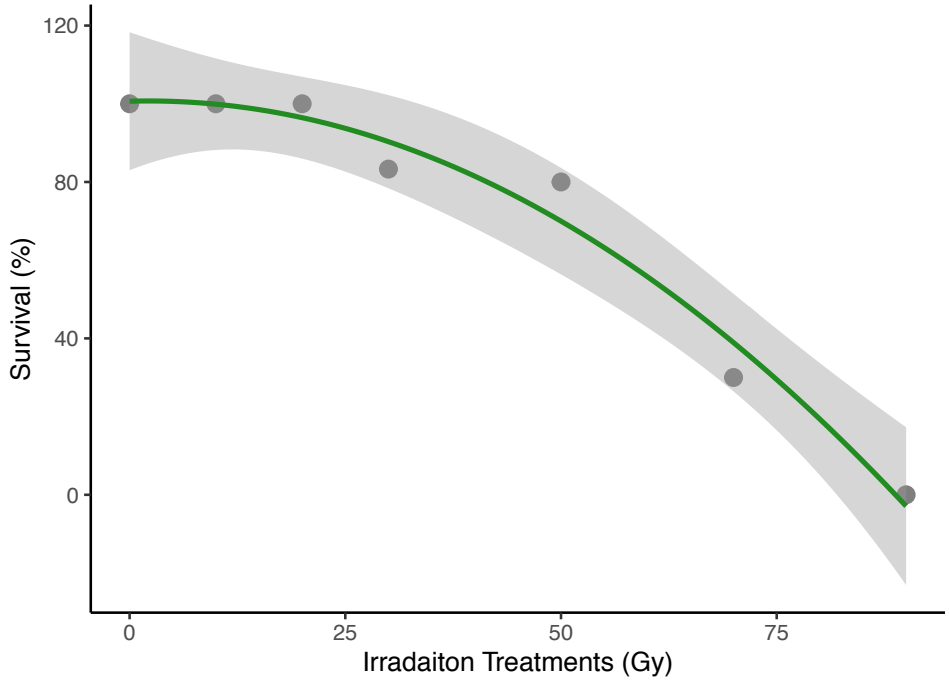

Figure C.2. The survival of *P.* virginianum at different doses of irradiation. The regression equation ($y = -0.0134x^2 + 0.058x + 100.66$; $R^2 = 0.9736$) predicts and LD_{50} rate at about 59.5 Gy.

APPENDIX D. A DISCUSSION OF *PYCNANTHEMUM* BREEDING EFFORTS

Introduction

Pycnanthemum is a relatively unexplored genus with potential as a marketable pollinator plant. Previous research has observed a greater abundance of pollinators visiting *Pycnanthemum* compared to other commonly known pollinator plants. MacLeod et al. (2020) found that *P. tenuifolium* attracted four times the crop-pollinating bees as *Asclepias tuberosa* and two times the number of rare bees as *Solidago rigida*. The study also emphasized that *A. tuberosa* and *S. rigida* were the second most visited plants by crop pollinating and rare bees, respectively, meaning *P. tenuifolium* attracted significantly more pollinators than all other genera in the study (MacLeod et al., 2020).

Previous breeding of *Pycnanthemum* has not focused on cultivar release, though hybridization has been used to compare compatibility between species. *Pycnanthemum* species are protandrous (Chambers & Chambers, 1971), meaning the male reproductive organs mature before the female reproductive structures. Protandry encourages outcrossing; however, *Pycnanthemum* inflorescences are composed of multiple flowers maturing at different rates, which allows self-pollination. Some plant genera have barriers to interspecific hybridization, limiting the exchange of genes between species. For example, embryo abortion, unreceptive stigma (Kuligowska et al., 2015), or geographical distance (Chambers & Chambers, 1971) can prevent crossing. *Pycnanthemum* hybrids are rare due to genetics and the distance between populations. Even in regions where different species overlap, plants are scattered, which limits the opportunity for cross-pollination.

The discovery of an apparent hybrid plant crossed between *P. virginianum* and *P. pilosum* (Chambers & Chambers, 1971) confirmed that natural interspecific hybridization was possible within the genus. Once determining the possibility of natural hybrids, Chambers (1993) made artificial crosses and examined the pollen stainability of the progeny. Pollen stainability is commonly used to infer the viability of pollen and, therefore, the compatibility between species. Chambers (1993) made crosses between *P. albescens*, *P. beadlei, P. californicum, P. curvipes, P. flexuosum*, *P. loomisii*, *P. montanum, P. muticum*, *P. nudum*, *P. pycnanthemoides*, *P. setosum,* and *P. tenuifolium*. Most of the crosses made in this study resulted in 0-30% stainable pollen. However, hybridizing *P. pycnanthemoides* with *P. beadlei* and *P. albescens* with *P. loomisii* yielded 31-56% stainable pollen (Chambers, 1993). The study explored compatibility between *Pycnanthemum* species, providing a foundation for future breeding programs.

Previous studies have attempted to predict the phylogeny of *Pycnanthemum* based on a variety of characteristics (Chambers, 1961a; Chambers & Chambers, 2008; Grant & Epling, 1943). Chambers and Chambers (2008) classified *Pycnanthemum* into sections (*Pycnanthemum*, *Aristatae*, *Brachystemum*, *Capitellatae*, *Macrocephalae*, *Nudae*, *Californicae*) based on morphology, chromosome number, ploidy level, and compatibility. Closely related plants are more likely to hybridize successfully (Harlan & de Wet, 1971); therefore, phylogeny can inform breeding decisions. The hybridization of species within sections should produce more viable progeny than those between sections.

Pycnanthemum (commonly known as mountain mint) is an herbaceous perennial member of the *Lamiaceae*. *Pycnanthemum* emits a mint or thyme-like fragrance when its leaves are bruised. The small, tubular white or purple flowers with purple speckles are arranged in a cyme. The genus is native to North America, with 18 species located in the central and eastern U.S. and

a single species native to California (Hummer et al., 2020). The 19 species include *P. albescens, P. beadlei, P. clinopodioides, P. curvipes, P. flexuosum, P. floridanum, P. incanum, P. loomisii, P. muticum, P. nudum, P. pilosum, P. pycnanthemoides, P. setosum, P. tenuifolium, P. torreyi, P. verticillatum, and P. virginianum*, and *P. californicum* (native to California)(Chambers, 1993). *Pycnanthemum*'s status as a native pollinator plant could support specialist pollinators and appeal to consumers looking for native plants. The genus also offers traits that, through cultivation, could appeal to consumers beyond the native plant market.

Cultivating *Pycnanthemum* must focus on improving characteristics related to aesthetics, maintenance, and pollinator resource availability. Consumers prefer low-maintenance plants with compact habits and showy flowers, while pollinators require high nectar production and viable pollen to provide sugars and protein (Yeamans et al., 2014). Breeding programs must focus on cultivating *Pycnanthemum* for consumers and pollinators to appeal to the public's movement toward plants with a purpose.

Materials and Methods

Plant Material

Propagation of seeds and cuttings occurred as germplasm was obtained (Table D.1). Ten seeds were planted per species, and ten cuttings were taken from each wild specimen for stock plants. Seeds were grown in a greenhouse with day/night conditions set to 25°C /20 °C and 40% / 30% humidity and allowed to germinate before being transplanted into 11.43 cm (956 mL) Kordlock Square Pots (The HC Companies, Twinsburg, GA). Seed germination and seedling growth took about 3 - 4 months before plants were ready for transplanting into C300 2.8 L pots (Nursery Supplies Inc., Kissimmee, FL) or the ground. Germplasm was also collected by botanizing, which involved taking cuttings from plants. Cuttings were treated with 3,000 mg/L

Indole-3-butyric acid (IBA) and inserted into 200 cell (14.6 mL) plug trays (Grower's Nursery Supplies, Salem, OR) with a 1:1 ratio of Pro-line C/B Growing Mix (Shady Dale, GA) to perlite. Cuttings were placed under 70% shade cloth with 21°C bottom heat and misted every five minutes for five seconds. Propagules had extensive root systems after two months. Plants were kept at the University of Georgia's Durham Horticulture Farm (33.944507, -83.375774). This site is a 90-acre farm located south of the University of Georgia and composed of Cecil sandy loam (USDA).

Year 1 selections were placed into the ground and irrigated using 20 mm wide drip tape with emitters spaced 30 cm apart (Rivulis Irrigation Ltd., San Diego, CA). In-ground plants were placed a meter apart in randomized complete blocks. The plot was fertilized once during the first year with Imperial Supreme 16-4-8 Lawn Fertilizer (16-1.8-6.6 N-P-K) (Athens Seed Lawn & Garden, Watkinsville, GA). Plants were watered three times a week for 90 minutes the first year and received only fain fall the second year. Pine straw was laid around plants and weeds were pulled by hand to combat weed pressure.

Plants selected during Year 2 were potted into C300 2.8 L pots (Nursery Supplies Inc., Kissimmee, FL) pots with an outdoor mix composed of 20% peat moss, 28% 0.95 cm aged pine bark, 42% 1.59 cm aged pine bark, and 10% sand (Old Castle, Shady Dale, GA), placed in a greenhouse, and received overhead watering. Plants in the greenhouse were fertilized with 24 g of Osmocote Plus 15-9-12, 8-9-month (15-4-10 N-P-K) (ICL Specialty Fertilizers, Summerville, SC). Plants were selected again in the greenhouse and selections were placed on a container pad, fertilized with an additional 24 g of Osmocote Plus, and watered twice daily via a sprinkler system.

Selection and Breeding

The germplasm was selected for round and compact growth habit, flower coverage, large flower size, purple flower color, and unique characteristics. Plants were also selected against lodging or dieback in the middle of the plants. All controlled crosses, open pollination, and self-pollination are listed in Table D.2 and D.3. Plants were open-pollinated and controlled crosses were made between species. After selection, plants were hybridized by removing flower heads from the male parent and pollinating flowers on the female parent. The crossing of species occurred without emasculation because *Pycnanthemum* species are protandrous (Chambers & Chambers, 1971). Controlled crossing and self-pollination were ensured by bagging flower heads before the flowers opened, while open pollination occurred naturally. Mesh bags were used before pollination and replaced after pollination to prevent bee pollination. Seeds were collected into separate bags during each seed collection and sown by cross into 11.43 cm (956 mL) pots with Pro-line C/B Growing Mix. Seedlings were separated into 200 cell (14.6 mL) plug trays after the cotyledons emerged to prevent competition between seedlings. Once the root systems were well established in plug trays, the plants were selected for compact growth habits and unique characteristics. The selected plants were potted into 6.65 cm (280 mL) Deep Press Fit Pots (The HC Companies, Twinsburg, GA) with Pro-line C/B Growing Mix and allowed to strengthen their root systems. Then, the plants were transplanted into C300 2.8 L pots (Nursery Supplies Inc., Kissimmee, FL) with the outdoor mix and grown on a container pad with overhead watering.

Seed germination was compared based on whether any seed germinated from a bag of collected seed. Percent germinations was then reported as the percent of seed bags that germinated to the total seed bags collected.

Results

Crosses during this study yielded a few F1 plants expressing unique characteristics. Selected plants will be grown for an additional year to provide a comprehensive understanding of growth and flowering traits in the F1 populations. *Pycnanthemum beadlei, P. incanum,* and *P. flexuosum* were the female parents with the lowest success (Table D.3). Open pollinated seed collected from *P. beadlei*, resulted in only 42.9% of the seed bags producing germinating seed. Only 25% of seed collections from open-pollinated *P. incanum* germinated. Similarly, 20% of seed bags collected from selfed *P. flexuosum* had seed that germinated. *Pycnanthemum flexuosum* also produced low germinating seed (40%) when the plant was the female parent of an interspecific cross. Open pollination was most successful for *P. muticum*, *P. pilosum*, and *P. virginianum* with 100% of the seed bags germinating (Table D.3). *Pycnanthemum tenuifolium* also successfully selfed with 100% of the seed collections germinating (Table D.3).

Discussion

Barriers to interspecific hybridization could explain the lack of germination in some *Pycnanthemum* crosses. Naturally occurring interspecific hybrids are rare due to geographical distance and genetic differences (Chambers & Chambers, 1971). *Pycnanthemum* hybrids have been found naturally (Chambers & Chambers, 1971) and produced artificially (Chambers, 1993), displaying the possibility of interspecific hybridization between *Pycnanthemum*. However, barriers to interspecific hybridization may reduce the success of crosses. Barriers exist both before and after fertilization. Pre-fertilization barriers affect the ability of plants to be successfully pollinated, while post-fertilization barriers prevent the formation of hybrid plants

(Kuligowska et al., 2015), which prevents successful seed formation or reduces the fertility of the F1 generation (Ling et al., 2022).

Ploidy level also acts as a barrier to hybridization. Plants with the same ploidy are more likely to cross and produce fertile progeny than when tetraploids cross with diploids. Hybridizing diploids and tetraploids produces triploids, which are generally sterile. Chambers (1993) reports germination was highest between *P. albescens* and *P. loomisii* (two diploids within the same sections according to Chambers and Chambers (2008)) and *P. pycnanthemoides* and *P. beadlei* (two tetraploids from different sections (Chambers & Chambers, 2008)). Therefore, an interaction between the ploidy level and phylogenic section could contribute to the hybridization success seen in the study.

Species should also crossing more readily within related groups than between groups, which could explain the success of *P. tenuifolium* and *P. virginianum* as parents. *Pycnanthemum tenuifolium* and *P. virginianum* were placed within proximity in the field, which encouraged hybridization. *Pycnanthemum muticum* and *P. pilosum* were also placed close to other diploid and tetraploid species within the *Capitellatae* section (Chambers & Chambers, 2008). Alternatively, *P. beadlei* may have experienced less successful hybridization due to the distance between *P. beadlei*, *P. floridanum,* or compatible *P. muticum* germplasm, which are all species Chambers and Chambers (2008) placed in the *Brachystemum* section. The breeding program only had a few *P. beadlei* and *P. floridanum*, limiting the hybridization of the plants by pollinators. *Pycnanthemum muticum* was present in the breeding program, though the germplasm obtained may have been diploid or pentaploid, which would limit successful crossing between plants. Similarly, *P. flexuosum* and *P. incanum* did not have many plants within their sections to facilitate hybridization.

P. tenuifolium and *P. flexuosum* were also bagged on five and four plants, respectively, to assess the potential for self-pollination. *Pycnanthemum tenuifolium* self-pollination resulted in 100% of all seed collections germinating, though 80% of the collections had less than 50% germination. *Pycnanthemum flexuosum* was not as successful when selfed. Four batches of seeds were collected, with 75% not germinating and the other 25% with less than 50% germination. Protandry limits self-pollination within *Pycnanthemum* (Chambers & Chambers, 1971). However, the differing success of seed formation seen in self-pollinated *P. flexuosum* and *P. tenuifolium* suggests barriers to self-pollination may vary across species in the genus.

Future studies should examine the success of reciprocal crosses to determine which species perform best as female and male parents. Self-compatibility should also be explored among all *Pycnanthemum* species to determine which species can self-pollinate successfully and examine recessive traits. Evaluating the products of hybridization between and within phylogenic sections and plants of varying ploidy levels will assist future breeding efforts. *Pycnanthemum* is a pollinator-attractive plant that could become an attractive addition to pollinator gardens. Determining the best parents for hybridization will inform future breeding programs and optimize cultivation.

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Tables and Figures

Table D.1. Table detailing the acquisition of germplasm used for the study.

Genus *Pycnanthemum*	Accession ID	Propagule	Collection Data
P. beadlei	CH2	cuttings	Stephens County, GA: Currahee Mountain; 1218'
P. beadlei	HL1	cuttings	Highland, NC: 3900'
P. flexuosum	W654808	seed	Pullman, WA: USDA-ARS Western Regional Plant Introduction Station
P. incanum	Inc SH	whole plant	Athens, Georgia: Mimsie Lanier Center for Native Plant Studies
P. muticum	RY1	cuttings	Watkinsville, GA: Durham Horticulture Farm
P. muticum	RY2	cuttings	Watkinsville, GA: Durham Horticulture Farm
P. muticum	RY3	cuttings	Watkinsville, GA: Durham Horticulture Farm
P. pilosum	Pilo SH	whole plant	Athens, GA: Mimsie Lanier Center for Native Plant Studies
P. tenuifolium	W654752	seed	Pullman, WA: USDA-ARS Western Regional Plant Introduction Station
P. tenuifolium	W654763	seed	Pullman, WA: USDA-ARS Western Regional Plant Introduction Station
P. tenuifolium	W657304	seed	Pullman, WA: USDA-ARS Western Regional Plant Introduction Station
P. virginianum	W657076	seed	Pullman, WA: USDA-ARS Western Regional Plant Introduction Station

Table D.2. List of the hybridization and seed bags collected in 2022. Types of crosses included open-pollinated seed (OP), selfing plants (☒), and interspecific crosses with the species listed as the female parent × male parent.

Cross No.	Genus *Pycnanthemum*	# of Seed Bags Collected	% Seed Bags that Germinated
1	*beadlei* (CH2) OP	2	0
2	*beadlei* (HL1) OP	4	75
5	*flexuosum* (W654808) × *flexuosum* (W654808)	1	0
6	*flexuosum* (W654808) × *pilosum* (pilo SH)	2	50
7	*flexuosum* (W654808) × *tenuifolium* (W657304)	2	50
8	*flexuosum* (W654808) ☒	4	25
9	*flexuosum* (W654808) OP	21	76
10	*incanum* (inc SH) OP	4	25
11	*muticum* (RY1) OP	3	100
12	*pilosum* (pilo SH) OP	4	75
13	*tenuifolium* (W654752) ☒	4	100
14	*tenuifolium* (W654752) OP	12	75
15	*tenuifolium* (W654752) x *pilosum* (pilo SH)	1	1
16	*tenuifolium* (W654763) ☒	1	100
17	*tenuifolium* (W654763) OP	4	75
18	*tenuifolium* (W657304) OP	6	100
19	*tenuifolium* (W657304) x *flexuosum* (W654808)	1	100
20	*tenuifolium* (W657304) x *pilosum* (pilo SH)	2	0
21	*virginianum* (W657076) OP	4	100

Table D.3. Table displaying the number of successfully germinating seed bags. Types of crosses included open-pollinated seed (OP), selfing plants (☒), and interspecific crosses with the species listed as the pistillate parent (X).

Genus *Pycnanthemum*	Cross	# of Bags from which Seed Germinated	# of Bags from which Seed Did Not Germinate	Successful Germination (%)
P. beadlei	OP	3	4	42.9
P. flexuosum	☒	1	4	20.0
	OP	16	5	76.2
	X	2	3	40.0
P. incanum	OP	1	3	25.0
P. muticum	OP	3	0	100.0
P. pilosum	OP	3	0	100.0
P. tenuifolium	☒	5	0	100.0
	OP	18	4	81.8
	X	2	2	50.0
P. virginianum	OP	4	0	100.0